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(Aus dem SCHÜFFNER-Laboratorium des Instituts für Tropische Hygiene, Amsterdam).

ÜBER EINE EPIZOOTIE UNTER ZAHMEN RATTEN UND DIE DAGEGEN GERICHTETE SCHUTZIMPFUNG DES PERSONALS

von

W. A. P. SCHÜFFNER und H. BOHLANDER

(Eingegangen am 5 Dezember 1940).

Unter dem Personal eines grossen Handels-Laboratoriums war ein Fall von Weil'scher Krankheit vorgekommen. Man vermutete, dass die Infektion mit dem Biss einer zahmen Ratte in ursächlichem Zusammenhang stand, und bat uns daher, den Ratten-Bestand der Firma auf Leptospirose zu untersuchen.

Man kann hierbei auf verschiedene Weise zu Werke gehen. Sind lebende Ratten zu untersuchen, und will man die Tiere erhalten, dann kann schon die Untersuchung des Urins im Dunkelfeld zum Ziele führen. Wenn man dies einige Tage hintereinander tut, kann man den grösseren Teil der Leptospirenträger herausholen. Diese Arbeit wird erleichtert, wenn man die Ratten auf Schutzstoffe mittels der Agglutinationsprobe bei einer Verdünnung des Serums 1 : 30 untersucht, und dann alle negativ reagierenden ausschaltet.

Ein erschöpfenderes Ergebnis erhält man bei Untersuchung der Niere nach Töten der Tiere. Bei älteren Prozessen — und um diese handelt es sich meistens — findet man die Leptospiren nesterweise in der Form eines Belages auf den Epithelzellen der Tubuli contorti; wenn man daher von der Nierenrinde Material entnimmt, hat man alle Aussicht, darin Leptospiren zu finden, vorausgesetzt, dass das Tier Träger war. Zum Auffinden der Leptospiren empfiehlt sich in erster Linie die Untersuchung des frischen Praeparates im Dunkelfeld. Es gelang uns dadurch 90% der Träger festzustellen; bei den übrigen 10% war die Kultur überlegen. Verimpft man Material der mikroskopisch negativen Tiere auf Meerschweinchen, dann kann das Resultat vielleicht um noch weitere Prozente verbessert werden. Aus Mangel an Tieren unterblieb das in unserm Fall.

Bei 50 Ratten der Gruppe, unter welchen sich das schuldige Tier befand, wurden 13 Tiere mikroskopisch und 2 durch Kultur als Träger gefunden. 42 weitere Ratten, als Stichproben aus den übrigen Ställen mit etwa 3000 Tieren entnommen, ergaben 17 bzw. 1 Träger, im Durchschnitt also 36% der erwachsenen Tiere. Damit war eine sehr erhebliche Enzootie unter dieser Rattenbevölkerung festgestellt. Man geht wohl nicht fehl, wenn man wilde Ratten, an denen in der Umgebung des Laboratoriums kein Mangel war, als Quelle der Infektion annimmt. Bei der Übertragung darf man den Kontakt mit Wasser, der die Infektion überwiegend beim Menschen vermittelt, ausschalten. An seine Stelle rückt der Kontakt mit dem Urin, und zwar entweder über die Nahrung oder direct durch Haut und Schleimhaute, wahrscheinlich auch durch den Geschlechtsact, wie es selbst beim Menschen vorkommen kann. Da die Leptospire eine aggressive Infektiosität besitzt, d.h. aus eigener Kraft den Weg in das Blut ihres Opfers findet, ist die starke Verbreitung der Infektion unter den Tieren, die so eng beieinander hausen, ohne weiteres begreiflich. Das Vorkommen solcher Enzootieen hat denn auch die frühere Auffassung, dass die „Weil" Infektion nicht kontagiös sei, aus der Welt geschafft.

Damit wurde zugleich die Lehre der sogenannten Umwandlung saprophytischer Leptospiren (*L. biflexa*) in hoch pathogene „Weil" Leptospiren getroffen. Diese Lehre stützte sich darauf, dass unter beinahe 100 Meerschweinchen, die mit einer *L. biflexa* eingespritzt wurden, schliesslich auch einmal eines der Tiere typisch an „Weil"erkrankte. Kontaktinfektionen, wie die eben beschriebene bei Ratten, waren damals noch unbekannt, und so konnte es geschehen, dass man zur Erklärung des einen „Weil"-Falles unter zahllosen negativen Experimenten nach der allen biologischen Regeln zuwiderlaufenden „Umwandlung" griff. Unsere zahlreichen eigenen Experimente bei denen die Möglichkeit eines unvorhergesehenen Kontaktes strengstens ausgeschaltet wurde, blieben ohne Zwischenfall. Dies negative, und darum an sich weniger überzeugende Resultat erhielt nun durch die Beobachtung der Enzootieen bei zahmen Mäusen (BESSEMANS) und zahmen Ratten (UHLENHUTH, KORTHOF), die nur mittels direkten Kontaktes entstehen können, eine kräftige Stütze; die Erkrankung des einen Tieres fand damit ihre Erklärung. Man muss eben damit rechnen, dass die „Weil" Infektion ganz unvermutet bei Menschen sowohl wie bei empfänglichen Tieren haften und dem Experimentator Possen spielen kann. Wir denken dabei auch an die zufälligen Infektionen von Kaninchen und die Störungen, die sie bei Untersuchungen anrichten können. Als Beispiel folgendes: In den ersten Jahren der Leptospirenforschung bezogen wir unser Kaninchen-Serum regelmässig vom Zentral Laboratorium in Utrecht, mit wechselndem Resultat.

Meist wuchsen unsere Kulturen darauf vortrefflich, aber dazwischen kamen auch Serumsendungen, auf denen nur einzelne unserer Stämme gediehen, andere geradezu abstarben. Wir schafften uns dann eigene Kaninchen an, um selbst das Serum mit allen Kautelen zu bereiten. Und in der Tat, der Erfolg war durchschlagend: alles wuchs üppig, bis nach etwa zwei Jahren sich der alte Übelstand aufs Neue geltend machte. Da diesmal zwei neu gekaufte Kaninchen gebraucht waren, kam uns der Gedanke, dass die Tiere spezifische oder unspezifische Antistoffe im Blute haben konnten. Ein Agglutinationsversuch brachte die Lösung: das eine Tier agglutinierte und lysierte den klassischen „Weil" bis zu einer beträchtlichen Titerhöhe; kein Wunder also, dass die Kulturen fast unmittelbar abstarben, soweit es sich um klassische Stämme handelte (heterologe Stämme gediehen dagegen). Seitdem wird bei uns jedes Tier, bevor es zu Kultur — oder, was ebenso wichtig ist — zu Immunisierungzwecken mit der einen oder anderen Art benützt wird, getestet. Im Laufe der Jahre haben wir auf diese Weise wiederholt Kaninchen ausschalten und uns so vor Katastrophen bezw. Irrtümern bewahren können.

Wir waren damit einer weit verbreiteten Enzootie bei dem fraglichen Rattenbestand auf die Spur gekommen, und es war fast verwunderlich, dass das Personal darunter nicht mehr zu leiden gehabt hatte. Jedenfalls bestand hier aber eine dauernde Gefahr und es war daher die nächste Frage, wie man ihr am besten begegnen konnte. Die Aufräumung des gesammten Rattenbestandes, mehr als 3000, kam nicht in Betracht, einmal der Kosten wegen und dann wegen der Unmöglichkeit, einen neuen Rattenbestand vor der Infektion zu schützen. Wir beschränkten uns daher auf allgemeine Vorschriften für das Umgehen mit den Tieren, deren Urin infektiös war, und schlugen als zweite Massregel die Schutzimpfung vor.

Auf Empfehlung der Japaner, später in Sumatra von Bärmann hatten wir früher schon unser eigenes Personal geimpft, sowie später das eines der hiesigen Universitäts-Institute. Dabei hatte sich herausgestellt, dass die Immunisierung bei intravenöser Injektion des Impfstoffes an wirksamsten war, wenigstens wenn man aus der Höhe des Titers der Agglutinations- und Lysis Probe Schlüsse ziehen durfte.

Der Impfstoff wurde zweimal gegeben, mit einem Abstand von einer Woche, das erste mal betrug die Dosis 1 ccm, das zweite mal 3 ccm. Etwa 4 Wochen nach der letzten Einspritzung wurde der Titer bestimmt. Die Tabelle gibt über die dabei erhaltenen Werte Aufschluss.

Tabelle

Sera	Stamm zahme Ratte	Stamm Kantorowicz	Stamm Wijnberg	Stamm Hond Utrecht IV
v. N.	300	> 100	300	0
Bl.	1000	> 100	300	0
Y. K.	> 300	30	300	0
G. L.	1000	> 100	1000	0
J. L.	> 300	300	300	10
E. W.	300	300	300	10
G. E.	300	300	300	100
Kij.	300	30	100	0
C. d. G.	300	300	300	10
Gr.	1000	100	300	0
A. Z.	1000	300	1000	0
A. d. J.	1000	300	300	0
H. S.	> 300	300	> 300	0
A. v. G.	1000	1000	1000	300
A. K.	1000	1000	1000	300
N. R.	300	300	> 300	30
W. v. G. krank gewesen Ende 1939	30000	10000	> 3000 (form)	> 300

Es sind hier die Werte für 3 klassische Stämme und die *L. canicola* angegeben. Der Stamm der weissen Ratten folgt fast genau dem Stamm „Wijnberg", der vollständigen oder A + B Biotype der klassischen Leptospira. Die unvollständige oder A Biotype bleibt bei 10 der 16 Personen in ihrer Titerhöhe zurück, und verrät dadurch ihren andersartigen antigenen Bau. *L. canicola,* als eine vollkommen heterologe Art, reagiert nur bei 7 der 16 Personen mit, der Titer ist bei 4 von diesen 7 kaum nennenswert und zeigt damit aufs Neue an, wie fern die *L. canicola* der klassischen Art steht.

Ob der erreichte Titer, der mit der Seroreaktion festgestellt werden konnte, auch einem entsprechenden Schutz entspricht, darf daraus natürlich nicht ohne weiteres abgeleitet werden. Der Beweis würde Kreuzversuche erfordert haben, für die es an den erforderlichen Cavias mangelte. Aber aus analogen früheren Versuchen wissen wir, dass die Höhe des Agglutinations-Lysis-Titers einen ziemlich zuverlässigen Massstab für die Immunisierung gibt. Eine weitere Stütze für die Wirksamkeit der Impfung sind die im grössten Massstabe und mit bestem Erfolg vorgenommenen Schutz-Impfungen unter den Bergleuten in Japan.

Zum Vergleich wurde der Titer des Reconvalescenten (ca 3 Monate nach dem Erkranken) beigefügt. Begreiflicherweise ist dieser beträchtlich höher wie der mit der Impfung erreichte. Die Reaktion mit dem Stamm „WIJNBERG" geschah hier mit formalinisierten Kulturen, die gewöhnlich ein bis zwei Verdünnungen unter der mit lebenden bleibt. Die Differenz zwischen der Reaktion mit dem Rattenstamm und .der WIJNBERG-Biotype will daher nichts besagen.

Auf Grund früherer von ESSEVELD durchgeführter orientierender Experimente wurde der Schutzstoff intravenös gegeben, eine Zuführung, bei der sich die Schutzstoffe schneller und reichlicher bilden. Dabei muss man allerdings leichtes Unbehagen bei einem Teil der Leute mit in Kauf nehmen. Sie waren indessen in keinem Falle so erheblich, dass von den Betroffenen die Arbeit hätte ausgesetzt werden müssen. Wohl kam es nach der zweiten Injektion fast unmittelbar bei drei der Geimpften zu einem leichten allergischen Shok, der sich in fliegender Röte von Gesicht und Hals, Hitzegefühl, leichtem Schwindel und beschleunigtem Puls äusserte, aber in 5 Minuten ohne weitere Folgen vorüber war. Um das zu vermeiden, werden wir bei neuen Impfungen die Kulturen ohne Pepton-Zusatz (1°/₀₀) wachsen lassen, und das eventuell minder gute Wachstum durch Anreicherung mittels Zentrifugieren ausgleichen. Ferner wird man gut tun, die zweite Injektion statt am 8. Tage, schon am 6. oder 7. Tage zu geben, wodurch sich eine Allergie vielleicht vermeiden lässt. Endlich bliebe zu erwägen, ob man mit einer Injektion schon genügenden Schutz erreicht, wenigstens soviel, dass dadurch die Gefahr eines tötlichem Ablaufs oder auch schon eines schweren Verlaufs vermieden werden könnte.

Neben allen besprochenen Massnahmen bleibt als vordringlichste Forderung, das Personal, die Arbeitgeber und den Hausarzt über die Gefahr, die die Laboratoriumsarbeit mit zahmen Ratten in sich birgt aufzuklären; vor allem der Arzt möge stets an die Möglichkeit einer Infektion denken. Wenn er darauf seine Diagnostik einstellt, dann wird es nicht vorkommen, dass die beste Zeit für eine wirksame Serotherapie ungenutzt verstreicht.

(From the „Centraalbureau voor Schimmelcultures", Baarn).

CULTURING OF MYXOMYCETES

by

H. A. DIDDENS [1]

(Received December 24, 1940).

Most of the studies on myxomycetes have been made with contaminated plasmodia and under uncontrolled conditions.

Therefore it seems important to discuss the methods of COHEN (2) who succeeded in the attaining of absolutely pure cultures of myxomycetes under controlled conditions. In his investigations he aims at a better understanding of the nutritional physiology of these organisms; the chief problems he attacks are: *a.* are myxomycetes able to feed saprophytically; *b.* which is the nutritional relation — if any — between the myxomycetes and their accompanying organisms.

I. PURIFICATION (ISOLATION).

Most of his cultures were obtained as plasmodia or sclerotia in the field, as the germination of spores may offer difficulties; moreover not all myxomycetes which are suitable for cultivation by their ability of forming readily plasmodia in culture, develop spores.

For the purification of the field material from the ever present contaminants (moulds, bacteria) two methods were used:

1. The migration method.
2. The enrichment method.

Before applying these methods the generally heavily contaminated collected plasmodia were cleansed of contaminants by allowing them to crawl over some plates of non-nutrient agar (2% agar). Thereupon a part of the plasmodium-bearing substrate was placed on moist filter paper in a petridish. As the plasmodium crept over the paper, oatmeal was sprinkled on or near it (modification of HOWARD's (3) culture-method), care being taken that not too many grains were added lest the contaminants get the upper hand. A piece of this filter paper bearing the plasmodium was transferred to a sterile agar plate.

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[1] Presented at the meeting of the „Nederlandsche Vereeniging voor Microbiologie", Utrecht, October 19, 1940; on that occasion COHEN's cultures, kept at the „Centraalbureau voor Schimmelcultures", were demonstrated. This paper gives a review of an article by A. I. COHEN (2).

1. In the migration method small pieces of plasmodium from this latter agar plate were placed in a plate with washed agar [1]) near the periphery. As soon as the crawling plasmodium had reached the opposite side of the dish without crossing its own track, it was transferred to another plate of washed agar; this process was repeated until the purity of the plasmodium was beyond all doubt. Generally three transfers sufficed for this purpose.

The migration method is effective by the starving of the contaminants on the very spot where they were placed on the extremely poor medium (washed agar), the plasmodium crawling away from them.

2. The second, the enrichment method, allows the myxomycete to feed and grow abundantly, whereas the contaminants are not supplied with sufficient nourishment. Washed agar plates were streaked with a suspension of washed living cells of *Saccharomyces ellipsoideus* Hansen [2]) (streaks 5 cm long); a piece of plasmodium (from the agar plate used preliminary to purification) was transferred at one end of the streak and after reaching the other end, inoculated again in a second similar plate, and so on. The living yeast cells serve as food only the myxomycete which can engulf them by means of its plasmodial strands, whereas they do not satisfy the needs of the undesired contaminating organisms.

The purity of the plasmodium in both methods was tested at each transfer by inoculating in yeast-extract broth [1]) and yeast-extract dextrose broth [2]).

---

[1]) 100 g granulated Difco Bacto agar are washed for 5 days with frequent changes of distilled water. The agar is squeezed out in fine cheese cloth untill all free water is driven out. From this agar 2% (dry weight basis) is prepared and buffered at pH 6.0 (KH_2PO_4/Na_2HPO_4 in a molar ratio of 8/2 : 1.45 g KH_2PO_4, 0.96 g Na_2HPO_4 (molar quantity being M/75 total PO_4).

[2]) The yeast is cultured in 1 l flasks containing yeast-extract dextrose broth (cf. note 2, page 9), without phosphate; from time to time the flasks are shaken for aeration. After 2 days the flasks are inclined until the yeast settles and the excess medium is decanted off through a flame. The yeast is washed several times with sterile distilled water. The final suspension is centrifuged and only a volume equal to that of the yeast layer is left.

When these broths remained clear, it was considered as a proof of the purity of the plasmodium. In case of the second method the yeast did not grow appreciably in the broth without dextrose.

Most contaminants (for the greater part bacteria) form nondescript spreading colonies in streak plates on the opaque, colourless media (washed agar, oatmeal agar); obviously in cultures along with the plasmodia they may be only — with difficulty — detected by careful microscopical and cultural examination. So the testing for purity by inoculating at regular intervals in yeast-extract broth is needed.

An agar-medium remaining clear is no sufficient proof of purity of a culture (cf. SKUPIENSKI (4)).

The migration method is simpler, gives on the whole better results than the enrichment method, but it may happen that a plasmodium is so completely devitalized by continued inanition that it can no longer be transferred successfully; then it may cross its own track and pick up contaminants again; moreover for want of food it may start fructification or sclerotization, either of them undesirable phenomena, as conditions influencing the germination of spores, a.s.o. are generally unknown.

The success of the enrichment method largely depends on the feeding rate of the plasmodium which must be rather fast. The too slow-feeding plasmodia may arrive at the end of the streak more heavily contaminated than at the moment of inoculation; moreover there is the danger of sclerotization. Fast-moving plasmodia are easily purified with both methods.

II. CULTURE.

Plasmodia purified by either methods described above, may be cultured in two ways:

1. in two-membered cultures („zweigliederige Kultur")
2. in pure cultures.

1. In two-membered cultures the plasmodia are feeding upon a second organism which develops on the supplied medium.

---

[1]) Yeast-extract (Difco, powdered) 2.5 g, tapwater 1 l. This broth is dispensed in tubes and sterilized by autoclaving 20 minutes at 15 pounds' pressure.

[2]) Similar to the preceding, with addition of 10 g dextrose and 1.4 g $Na_2HPO_4 . 12H_2O$.

In most of his experiments COHEN used *Saccharomyces ellipsoideus* Hansen as second organism, because this yeast is fast-growing and neither too small, nor glutinous. Some other yeasts and moulds tested lack these advantages.

The pure plasmodium was transferred into oatmeal agar [1]) flasks with *S. ellipsoideus*. This medium allows the yeast to grow moderately, whereas richer media yield too heavy a growth of yeast.

When the isolation was made by enrichment method it seldom was necessary to inoculate the yeast, as the plasmodium carried sufficient yeast with it.

2. For pure culture the purified plasmodium was streaked with the autoclaved bakers' yeast [2]) — in the same way as the living yeast was streaked in the enrichment method — on washed agar slants. Other media tried (plugs of potato, carrot, banana, apple, oatmeal agar, yeast-extract agar, pea agar, etc.) gave negative results. The good results on the washed agar medium are in entire agreement. with the good results of the two-membered cultures.

Plasmodia isolated by enrichment method were freed from the yeast by some migrations over washed agar.

Beside the claim of purity COHEN insisted on the need that the cultures should not decline in vigour through many transfers, or — better still — should show good growth indefinitely.

He was successful in culturing: *Badhamia foliicola* Lister; *B. utricularis* (Bull.) Berk., *Didymium squamulosum* Fr., *Fuligo septica* (L.) Weber, *Physarum polycephalum* Schw. and *Stemonitis axifera* Macbride in pure culture.

III. STORAGE OF CULTURES.

Myxomycete-cultures ask for a good deal of transferring, as various products of wastage soon injure the vitality of the plasmodia. Pure cultures were kept at 25° C. and the gross and two-membered cultures at room temperature.

---

[1]) Quaker rolled Oats is dispensed into 125 ml Erlenmeyer flasks to an even layer one grain in thickness; 20 ml unwashed agar (pH 6.0, cf. washed agar) is poured into each flask. Sterilization by autoclaving 20 minutes at 15 pounds' pressure.

[2]) Equal quantities by weight of bakers' yeast and distilled water are well mixed in a mortar and dispensed into tubes in 5 ml quantities for autoclaving (20 minutes at 15 pounds' pressure). Before use the tubes are to be shaken.

In order to cut down the large number of transfers, cultures often were sclerotized by the method of HOWARD (3), a slow drying process, and kept in envelopes in the refrigerator. Not all these experiments proved to be successful. A sclerotium, however, on moistening may change within a few hours into a fast-crawling plasmodium.

From COHEN's experiments it is evident that the plasmodium and yeast are not in competition for the nutritive medium (a view brought forward by CHRZASZCZ (1)); neither seem metabolic products of the yeast or the secreted slime to serve as nutrients, as yeast cells are seen to be engulfed and destroyed by the plasmodium.

Moreover his studies show that the growth of myxomycetes is greatly influenced by temperature, size of inoculum, pH, etc., factors which may change the balance between plasmodium and its co-organism in two-membered cultures.

The foregoing may have given an impression of the useful piece of work done by COHEN in this rather neglected field of botany; he succeeded in cultivating in pure culture several myxomycetes which offer difficulties in handling.

In view of the results arrived at already by COHEN, one hopes that this author may continue his experiments.

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BEOBACHTUNGEN AN EINER KULTUR VON *ENTAMOEBA HISTOLYTICA* (SCHAUDINN)

von

FRANZ PICK

(Eingegangen am 15 September 1940).

Seit der sicheren Abtrennung der Dysenterieamöbe durch SCHAUDINN (19) und der ordnenden Arbeit DOBELLS (7, 8, 9) und HARTMANNS (13) in der komplexen Amöbenfrage, war ein langer Weg zurückzulegen; die Namen LÖSCH (16), COUNCILMAN und LAFLEUR (5), BRUG (3, 4), KUENEN (14), VON PROWAZEK (17), SWELLENGREBEL (15) u.a. bleiben dauernd mit der Geschichte der Dysenterieamöbenforschung verbunden. Erst spät gelang es brauchbare Nährböden zu finden. Die Entdeckung LAIDLAWS (9) bedeutete einen gewaltigen Fortschritt in der Frage der Kultivierung der Entamöben. Die Wichtigkeit des Zusatzes von Reisstärke ist heute allgemein anerkannt. Mitunter kann aber doch der Fall eintreten, dass auch bei Verwendung erprobter Nährböden, mit jeweiligem Stärkezusatz, und obschon eine grössere Anzahl guter Subkulturen erzielt werden konnte, die Stämme zurückgehen und allenfalls ganz verschwinden. Unsere Untersuchungen wurden an einem Stamm ausgeführt, der im ,,Instituut voor Tropische Geneeskunde" in Leiden, von mir als A.F. 13. bezeichnet worden war.

1. EINFLUSS DES NÄHRBODENS.

Der Stamm A.F. 13. hatte bis zum Zeitpunkte des Anfanges der Untersuchungen eine Reihe von Subkulturen ergeben auf einem Nährboden vom Typus DOBELL (10) und war im Zurückgehen. Dieser Nährboden enthält:

Als festen Bestandteil: Koaguliertes Pferdeserum, als flüssigen Bestandteil: Ringer-Eiweiss. Die Herstellung erfolgte nach folgender Vorschrift. Pferdeserum wird steril zu je 5 ccm in Reagensröhrchen abgefüllt und kommt darauf in Schräglage durch 60 bis 75 Minuten bei 80° C. in den Seumofen. Ferner werden 250 ccm Ringerflüssigkeit und 9,5 ccm

Eiweiss unter den Bedingungen der üblichen Sterilität, in einer Flasche mittels Glasperlen gut gemischt. 3 bis 4 ccm dieser Ringer-Eiweiss Flüssigkeit werden zu je einem Reagensröhrchen mit dem nun koagulierten Pferdeserum gefügt. Hierauf erfolgt die übliche Prüfung der so beschickten Röhrchen, auf den Behalt der Sterilität.

Zur Regenerierung des Stammes A.F. 13. wurde zuerst ein Ansetzen auf einem anderen Nährboden, nämlich auf Bacto Liver Infusion Agar versucht.

Originalvorschrift:

Beef liver, Infusion 500 g, Proteose, Peptone (Difco) 10 g, Sodium Chloride 5 g, Agar (Bacto) 20 g.

The Bacto Liver Infusion Agar is prepared for use by dissolving 55 g of the powder in 1000 ccm cold distilled water. Boil a few minutes to dissolve the agar. Dispens as desired and sterilize 20′ at 15 pounds pressure. The „Growth Accessory Factors" are retained.

Nach obiger Vorschrift wurde in je ein Meinicke-Röhrchen 1 ccm des Liver Infusion Agar gebracht, nochmals sterilisiert und nach dem Erstarren steril je 2 ccm Pferdeserum-Ringer pro Röhrchen zugefügt. Die Ergebnisse dieser Kultivierungsversuche waren nicht zufriedenstellend, da auch durch das dreimalige Übertragen des Stammes A.F. 13 auf einen anderen Nährboden keine Erholung desselben erzielt werden konnte.

Es ist nun oft zu beobachten, dass die Amöben beim Durchlaufen auch zahlreicher Passagen Schwankungen in ihrer Wachstumsdisposition unterliegen. Die Abnahme der Amöben an Zahl kann schliesslich soweit führen, dass die Kulturen als vollkommen negativ bezeichnet werden müssen. Man kann aber auch nach einer zahlenmässigen Verminderung ein plötzliches Aufflackern der Kulturen beobachten, das manchmal auch vom Auftreten von Riesenamöben begleitet sein kann, was als besonders gutes Zeichen des Gedeihens einer Kultur bewertet wird. Eine solche verschiedenartige Disposition im Wachstum der Amöben kann nicht auf äusseren Umständen beruhen, denn auch bei Gleichmässigkeit in der Zusammensetzung des Nährbodens, Konstanz der Temperatur und der begleitenden Bakterienflora kann eine solche Änderung eintreten. Es war daher naheliegend, diesen Schwankungen in den Amöbenkulturen andere Ursachen zu Grunde zu legen und an Faktoren zu denken, die auch bei genauester Zubereitung, in den Nährböden selbst in verschiedenem Ausmasse oder in verschiedener Stärke vorhanden sein konnten.

Es war zu überlegen, ob hier nicht wachstumshemmende bezw.

wachstumsfördernde Faktoren eine Rolle spielen. Wenn man die Zusammensetzung der beiden oben angegebenen Nährböden betrachtet, findet man in beiden Serum, in dem zweiten auch noch Leber. Sowohl in der Leber (20) als auch im Serum (1) sind wachstumshemmende Stoffe vorhanden, die überdies mit dem Alter der Tiere zunehmen. Daraus könnte man ungezwungen die Schwankungen bei der Kultivierung der *Entamoeba histolytica* erklären. Hier ist auch ein Bericht von BRUG (3) zu erwähnen, der bei der Kultivierung von *Entamoeba histolytica* auf dem serumfreien Nährboden von GUERIN und PONS (12) ziemlich gute Resultate erzielt hat, aber nach Zusatz von steriler Mäuse- oder Meerschweinchen-Leber zu den Kulturen, wurden die Amöben nach 2—3 Tagen getötet. Ebenso tötete Meerschweinchen-Leber, die auf 100° C. erhitzt wurde, die Kulturen in 2—3 Tagen. Auch das spricht für die Wirkung von wachstumshemmenden Stoffen, da zu dem letzten Versuch BRUGS zu bemerken ist, dass die wachstumshemmenden Stoffe der Leber auch bei 100° C. nicht zerstört werden.

Aus dem bisher Gesagtem ergibt sich, dass der Versuch zu unternehmen war, das Gleichgewicht zwischen den wachstumshemmenden und den wachstumsfördernden Faktoren sicherzustellen oder, wenn es gelingen könnte, zu Gunsten der wachstumsfördernden Stoffe zu ver-

Tabelle 1.

Versuch vom 13. IV.—15. IV. 1940 mit Zusatz von Insulin.

Kulturen Nr.	Zusatz	Ablesung
I	1 Tr. Insulin	+ +
Ia	1 Tr. ,,	+ +
II	3 Tr. Insulin	+ + +
IIa	3 Tr. ,,	+ + +
III	6 Tr. Insulin	+ +
IIIa	6 Tr. ,,	+ +
IV	9 Tr. Insulin	+
IVa	9 Tr. ,,	+
V	12 Tr. Insulin	(+)
Va	12 Tr. ,,	(+)
VI	15 Tr. Insulin	—
VIa	15 Tr. ,,	—
VII	An Stelle des flüssigen Nährbodens: 2 ccm reines Insulin (80 int. E.)	—
VIIa	Derselbe Zusatz	—
K	Kein Insulinzusatz	+
Ka	,, ,,	+

Legende:
— = negativ.
+ = vereinzelte Amöben.
+ + = mehrere Amöben.
+ + + = viele Amöben.
+ + + + = sehr viele Amöben.
+ + + + + = Amöben so zahlreich, dass sie praktisch nicht zählbar sind.
R = vereinzelte Amöben mit einem Stärkekorn.
RR = bessere Stärkekörneraufnahme.
RRR = gute Stärkekörneraufnahme.

schieben. Untersucht wurde ob Zusatz von Insulin eine Änderung im Wachstum auslösen würde.

Methodik der Kultivierung: Liver Infusion Agar und Pferdeserum-Ringer. Meinicke-Röhrchen mit je einem ccm festem und 2 ccm flüssigem Nährboden. Der zurückgehende Stamm wurde neuerdings übertragen, aber diesmal unter Beifügung verschiedener Mengen Insulin (Verwendet wurde Insulin ,,Organon", 40 int. E. pro 1 ccm). Allen Röhrchen einschliesslich der Kontrollen, wurde je 2 Ösen Reisstärke und je eine Öse $CaCO_3$ zugesetzt. Tabelle 1 ergibt die Resultate.

Aus diesem Versuch erhellt, dass zu dem Zeitpunkt der Versuchsreihe der Zusatz von 3 Tropfen Insulin am geeignetesten war. Daher wurde der nächste Versuch mit den Kulturen II und IIa fortgesetzt, indem die angelegten Subkulturen je 3 Tropfen Insulin erhielten, während die übrigen Versuchsbedingungen unverändert blieben. In den Kulturen vom 15. IV. — 17. IV. bei einem Zusatz von 3 Tropfen Insulin erhielten sich die Amöben sehr mobil. Eine grosse Zahl von Amöben liegt in ,,Nestern" beisammen. Die Amöben der Kontrollkulturen haben verhältnismässig viel Stärke aufgenommen.

Man sieht bereits in der zweiten Subkultur einen beträchtlichen Unterschied zwischen den ,,Insulinamöben" und denen der Kontrollkulturen. Auch nehmen die Insulinamöben die Stärkekörner reichlicher auf.

Die Nesterbildung ist besonders im Bereiche von verkohlten Wattefäden, die beim Aufsetzen der abgebrannten Wattepfropfen in die Kultur gefallen sind, zu beobachten. Der nächste Versuch wird in diesem Sinne erweitert um das Zufallsmoment auszuschliessen (Tabelle 2).

Tabelle 2.

Versuch vom 17. IV. — 20. IV. 1940. Zusatz von verkohlten Wattefäden.

Kulturen Nr.	Zusatz	Ablesung
I	3 Tr. Insulin	+ + + RR
Ia	3 Tr. ,,	+ + + RR
II	3 Tr. ,,	+ + + RR
IIa	3 Tr. ,,	+ + + RR
III	3 Tr. Insulin plus verkohlte Watte	+ + + + RR
IIIa	3 Tr. Insulin plus verkohlte Watte	+ + + + RR
IV	3 Tr. Insulin plus verkohlte Watte	+ + + + RR
IVa	3 Tr. Insulin plus verkohlte Watte	+ + + + RR
K	—	+ + RR
Ka	—	+ + RR

Bei den Kulturen III, IIIa, IV und IVa ergibt sich die Bildung von zahlreichen Nestern, besonders im Bereiche der verkohlten Wattefäden. Diese Nester werden von Amöben gebildet, die dicht beieinander liegen als wenn sie ,,agglutiniert" wären. Man hat den Eindruck, ein Stück eines Gewebes vor sich zu haben. Beim Abimpfen der Kulturen ist eine schleimig-fadenziehende Konsistenz der Stärke zu beobachten. Hieraus ergibt sich eine neue Versuchsanordnung. Bei dem folgenden Versuch wird die Stärke nicht mehr wie bisher trocken zugesetzt, sondern in Form eines Insulin-Stärke-Breies, in dem die Stärke in dem Insulin suspendiert ist. Auf je einen Tropfen Insulin wird eine Öse Stärke gegeben. Die Zubereitung geschieht am besten so, dass man in 10 Tropfen Insulin 10 Ösen Stärke suspendiert und mit der Pasteurschen Pipette gut mengt. Mit dieser Pipette wird dann die Suspension aufgesaugt und auf den Grund des flüssigen Nährbodenbestandteiles eine etwa 1 mm hohe Lage aufgetragen, was bei einiger Vorsicht ohne Wolkenbildung geschehen kann. Die Lage von etwa einem mm entspricht in der Flüssigkeitsmenge ungefähr 3 Tropfen Insulin. Es empfiehlt sich, auch aus Gründen der Sterilität, diesen Insulinbrei jedesmal neuzubereiten, wobei man das entsprechende Quantum $CaCO_3$ ebenfalls beimengen kann (Tabelle 3).

Tabelle 3.

Versuch vom 20. IV. — 22. IV. 1940. mit in Insulin suspendierter Stärke.

Kulturen Nr.	Zusatz	Ablesung	
I	3 Tr. Insulin	+ + +	RR
Ia	3 Tr. ,,	+ + +	RR
II	3 Tr. ,,	+ + +	RR
IIa	3 Tr. ,,	+ + +	RR
III	3 Tr. Insulin plus verkohlte Watte	+ + + +	RR
IIIa	3 Tr. Insulin plus verkohlte Watte	+ + + +	RR
IV	3 Tr. Insulin plus verkohlte Watte	+ + + +	RR
IVa	3 Tr. Insulin plus verkohlte Watte	+ + + +	RR
V	3 Tr. Insulinbrei	+ + + + +	RRR
Va	3 Tr. ,,	+ + + + +	RRR
VI	3 Tr. ,,	+ + + + +	RRR
VIa	3 Tr. ,,	+ + + + +	RRR
K	—	+ +	R
Ka	—	+ +	R

Die Amöben liegen in der Insulinbrei-Kultur in zahlreichen Nestern zu vielen Hunderten wie ,,agglutiniert'' beisammen und verdecken ganze Gesichtsfelder. Eine Auszählung der Kulturen ist undurchführbar. Die Gesamtzahl in einer so kleinen Kultur in einem Meinicke-Röhrchen geht in die vielen Tausende, wahrscheinlich in einige Zehntausende. Zum Vergleich sei hier auf die Ergebnisse DOBELLS (7) verwiesen, wobei zu bemerken ist, dass DOBELL aller Wahrscheinlichkeit nach, den auch der Form nach unveränderten Nährboden von BOECK-DRBOHLAV verwendet hat, also mit viel grösserer Nährbodenfläche arbeitete, im Vergleich zu dem kleinen Querschnitt eines Meinicke-Röhrchens.

Aus seiner Tabelle ergibt sich ein Durchschnittsgehalt der Kulturen an Amöben von ungefähr 600 je Kultur, demnach nicht annähernd die Zahl der Amöben, die aus einer Insulinbrei-Kultur in einem einzigen kleinen Kulturtropfen enthalten sind.

Die weiteren Subkulturen vom 22. IV. — 8. V. haben mit einigen Schwankungen einzelner Kulturen, die zu lange einer niedrigen Temperatur ausgesetzt waren, in gleichmässiger Weise, dieselben Ergebnisse gezeitigt.

2. FÄRBERISCHE VITALITÄTSPRÜFUNG UND VITALFÄRBUNG.

Die Vitalitätsprüfung nach Swellengrebel (15) mit 2% wässriger Eosinlösung, liess bei allen Kulturen nur vereinzelte tote Amöben erkennen.

Beginnend mit der Ablesung vom 26. IV. zeigten alle Kulturen ohne Ausnahme bei der Färbung nach Delafield (18) das Vorhandensein zahlreicher ein- bis zweikerniger Cysten mit gut ausgebildeten Chromidialkörpern. Durch geringfügigen Zusatz von Pyocyanin gelingt es eine Vitalfärbung der Amöben zu erreichen. Es färben sich aber auch die toten Amöben. Die Lebenden, die eine etwas leuchtend grünliche Färbung annehmen, sind dadurch etwas besser sichtbar. Auch scheint der Farbstoff ihre Beweglichkeit zu erhöhen. Zusatz grösserer Mengen von Pyocyanin tötet die Amöben bevor eine Änderung in der begleitenden Bakterienflora auftritt.

Das Pyocyanin ist jedenfalls nicht ohne weiteres geeignet, um die Kulturen der Amöben bakterienärmer zu machen, denn es wurden auch hier die Beobachtungen und Erfahrungen von Brug (3) bestätigt, der versucht hat durch verschiedene Mittel darunter auch Kupfersulfat, die Kulturen von den Bakterien zu befreien, um z.B. die Cysten rein darzustellen. Bemerkenswert ist auch ein Versuch von Deschiens (6) mit Trypaflavin (3 gouttes d'une solution à 1 p 1.000 pour 5 cc d'eau d'égout). Deschiens sagt weiter nichts über diesen so durchdringenden Farbstoff aus, als dass er in seiner Wirkung als Antisepticum die Vermehrung der Mikroben herabsetzt und dabei noch 3 Überimpfungen der Kultur-Amöben gestattet. Das Pyocyanin penetriert alles, da sich aber in unserem Falle die Amöben damit imbibieren, grünlicher erscheinen und vielleicht sogar etwas an Beweglichkeit zunehmen, so sind damit die Forderungen an eine Vitalfärbung erfüllt.

3. POLARISIERUNG DER *ENTAMOEBA HISTOLYTICA*.

In jeder Kultur mit beweglichen Amöben kann man beobachten, dass die Amöben häufig einen mehr oder minder breit aufsitzenden Detritusklumpen mitschleppen. Manchmal macht es auch den Eindruck, dass die Amöben sich von diesem Ballast befreien wollen. Es ist nicht zu entscheiden, ob es sich hierbei um die Klebrigkeit des Ektoplasmas handelt, wodurch ein Hängenbleiben von Detritus an irgendeiner Stelle der Amöbenoberfläche möglich ist, oder ob es sich dabei tatsächlich um einen Pol der Amöbe handelt. Da ein histologischer Beweis kaum zu erbringen war, musste nach einem funktionellen Beweis gesucht werden. Dazu wurde, nach Beobachtungen innerhalb des Rahmens einer anderen Arbeit, defibriniertes Schafsblut verwendet.

Versuchsanordnung: Auf einen Objektträger wird ein Tropfen aus einer Amöbenkultur gebracht und eine kleine Öse defibriniertes Schafsblut zugesetzt. Man achte darauf, dass nur so wenig zugesetzt wird, dass die Erythrozyten das Bild nicht vollkommen verdecken. Die Beobachtung erfolgt am besten auf einem geheizten Objekttisch bei 33° C. Es empfiehlt sich Insulinamöben zu nehmen, die aus einer frischen Kultur stammen, da sie dann sehr jung und beweglich sind, daher auch funktionelle Eigenschaften am besten zeigen.

Wenige Sekunden nach Beginn des Versuches kann man beobachten, dass einzelne Amöben trotz guter Stärkeaufnahme noch je einen Erythrozyten einschliessen, was als Zeichen besonders starker Vitalität angesehen werden kann.

Bei weiterer Beobachtung sieht man, wie die inzwischen stechapfelförmig deformierten Erythrozyten, mit einem ihrer Fortsätze von den Amöben angezogen werden. Diese Anziehung der Amöben geht von einem Punkt aus und wird gleichzeitig auf mehrere Erythrozyten ausgeübt, die sich an diesem Punkt zu vereinigen suchen. Die Erythrozyten werden nun mit ihren Fortsätzen zusammengefasst und bouquet-artig miteinander verbunden, wobei es zu einer elliptischen Deformierung der Erythrozyten kommt. Die Anziehungskraft, die von diesem Punkte ausgeht, ist damit aber keineswegs erschöpft, sondern es ordnet sich an der Breitseite des Bouquets eine zweite Lage von Erythrozyten an, wobei die Fortsätze der Erythrozyten der zweiten Lage, zwischen die schon an die Amöben fixierten Erythrozyten hineingezogen werden. Es kommt dann noch zur Ausbildung einer dritten Lage von Erythrozyten im analogen Sinne. Die elliptische Deformierung der Erythrozyten der zweiten Lage ist geringer, als die der direkt der Amöbenoberfläche aufsitzenden Erythrozyten und ist in der dritten Lage kaum noch zu bemerken. Nachdem das Erythrozyten-Bouquet vollkommen ausgebildet ist, kann man auch weiterhin die Anziehung beobachten, die von dem Punkte der Amöbenoberfläche ausgeht. Einzelne Erythrozyten suchen noch in tanzender Bewegung sich in das Bouquet einzuordnen, ohne jedoch Platz zu finden, und werden wieder abgestossen. Die Erythrozyten werden demnach nicht nur von dieser bestimmten Stelle der Amöbenoberfläche angezogen, sondern auch dadurch in einer bestimmten Anordnung vereinigt. Diese Anordnung, die bei zahlreichen Amöben anzutreffen ist, muss aber nicht immer so typisch sein und man sieht dann in diesen Fällen, dass die Amöben die Erythrozyten wohl anziehen aber nicht zu einem Bouquet ordnen können. In diesen Fällen liegt, über ein grösseres Feld der Amöbenoberfläche verteilt, eine grössere oder kleinere Anzahl von Erythrozyten, wobei es höchstens zur Ausbildung einer schwach besetzten zweiten Lage kommt. In diesen Fällen sitzt nicht der Stiel eines Bouquets mit seiner Spitze den Amöben auf, sondern es wird ein kleiner Teil der Oberfläche kuppenartig überdeckt. Gegenüber toten Amöben oder deren schon ganz amorphen Überresten zeigen die Erythrozyten dasselbe Verhalten wie gegen andere zellige Elemente oder den vorhandenen Detritus.

Ganz ungeordnet, teils neben, teils über diesen zelligen Elementen liegend, zeigen die Erythrozyten nicht die geringste Änderung, in Lage, Form oder Bewegung.

Die Bewegung der Amöben ändert sich, wenn diese ein so grosses Erythrozyten-Bouquet hinter sich nachschleppen. Eigentlich müsste man eine Streckung des Amöbenkörpers in der Fortbewegungsrichtung erwarten. Es tritt aber das Gegenteil ein. Es kommt zu einer Verbreiterung des Amöbenkörpers, dessen längere Achse zu der Bewegungsrichtung senkrecht steht. Auch die Ausbildung der Pseudopodien hat sich geändert, denn es werden jetzt fast gleichzeitig 2—3 Pseudopodien ausgestülpt, von denen eines bestehen bleibt und die Richtung angibt, während die anderen sich wieder zurückziehen. Die Vorwärtsbewegung ist infolge des grossen Ballastes schleppend. Die Verbindung zwischen Amöben und Erythrozyten scheint sehr stark zu sein, da niemals eine Trennung beobachtet werden konnte. Bei vorsichtiger Färbung nach Delafield gelingt es die Erythrozyten-Bouquets in ihrer Verbindung mit der Oberfläche der Amöben, färberisch darzustellen.

Das Verhalten der Erythrozyten gegenüber den Cysten ist ebenfalls eigenartig. Die Erythrozyten werden von den Cysten angezogen, aber gleichzeitig in einem geregelten Abstand gehalten, so zwar, dass ein konzentrischer Hof um die Cysten entsteht. Dadurch werden die Cysten schon optisch deutlich aus der übrigen Umgebung hervorgehoben.

Zum Abschluss wäre noch auf eine Erscheinung hinzuweisen, die möglicherweise mit der Vorbereitung der Excystierung in Zusammenhang gebracht werden kann. In ganz vereinzelten Fällen konnten an Amöben, die ebenfalls aus einer Insulinkultur stammten, folgende Erscheinungen beobachtet werden. Eine ruhig und abgerundet daliegende Amöbe streckt ziemlich rasch ein Pseudopodium aus, ohne dasselbe wieder zurückzuziehen oder das Entoplasma nachströmen zu lassen. Das so ausgestreckte Pseudopodium dellt sich an der Kuppe ein und beginnt sich gleichzeitig um seine Basis zu drehen. Nachdem die Stieldrehung beendet ist, beginnt das Pseudopodium in dieser Haltung um die Zirkumferenz der Amöbenoberfläche zu wandern, wobei es immer kleiner wird, um schliesslich, am Ausgangspunkt angelangt, wieder mit der Oberfläche zu verschmelzen. Wenn man berücksichtigt, dass die Stieldrehung nicht aufgegeben wird, daher zwischen dem abgedrehten Ektoplasma und dem übrigen Teil desselben, eine Kommunikation bestehen bleibt und an die grosse Dehnbarkeit und Elastizität des Ektoplasmas denkt, wäre vielleicht der Schluss gerechtfertigt, dass es sich hier um einen vorbereitenden Prozess der Excystation handelt. Eine ähnliche Erscheinung hat schon vor langer Zeit Brug (3) beschrieben. Wenn man die von mir beschriebene Erscheinung eines stielgedrehten Pseudopodiums in der Durchsicht betrachtet und die Eindellung der Kuppe in ihrer Projektion festhält, so hat es tatsächlich eine gewisse Ähnlichkeit mit der von Brug abgebildeten Figur

4. DISKUSSION.

Die Beimengung eines Insulin-Stärke-Gemisches zu Kulturen von *Entamoeba histolytica* bewirkt ein sehr reichliches Wachstum der Amöben. Das Auftreten zahlreicher Riesenamöben ist ein weiteres Zeichen für das Gedeihen der Kulturen ebenso das Erscheinen von Cysten mit gut ausgebildeten Chromidialkörpern. Die Ursache dieser Erscheinungen dürfte in einer Vermehrung wachstumsfördernder Stoffe liegen. Die Nesterbildung in Ausmassen, die ganze Gesichtsfelder verdecken, spricht wohl für eine gelungene Nachahmung günstiger Verhältnisse für die Amöben, wie sie in den Fundi der Drüsen und bei Geschwürsbildung bei Amöbeninfektion angetroffen werden, wobei es fast typisch zur Nesterbildung kommt.

Für die Praxis scheint mit dem Zusatz von Insulin ein Verfahren gefunden zu sein, das durch die Anreicherung von Amöben, die Diagnose vereinfacht und erleichtert. Kommt es ja gerade bei der Amoebiasis auf die Sicherung der Diagnose innerhalb kürzester Zeit nicht an. Man hat also unter den durchschnittlichen Bedingungen einer Erkrankung an Amöbenruhr, Zeit, um eine Kultur, allenfalls noch ein oder zwei Subkulturen anzulegen.

Ob es zweckmässig wäre, überdies den Patienten selbst Insulin zu geben, um die Amöben zu diagnostischen Zwecken zu grösserer Vermehrung zu veranlassen oder dadurch auch eine Mobilisierung der

Amöben anzustreben, in dem Sinne, dass jugendliche Formen leichter therapeutisch angreifbar sind, müsste noch untersucht werden. Es würde sich dabei natürlich nur darum handeln, für kurze Zeit eine Vermehrung der Amöben zu forcieren, um dadurch eine Spontan-Mobilisierung zu erreichen.

In diesem Zusammenhang sei an die Beobachtungen von **Ghosch** (11) u.a. erinnert, wonach die praktische Unmöglichkeit die Amoebiasis in Indien zu beseitigen, auf die besonders kohlehydratreiche Kost der Indier zurückgeführt wird. Ob es wie **Ghosch** vermutet, die saure Reaktion des Darminhaltes ist, die eine Vermehrung der Amöben begünstigt, erscheint nach obigen Untersuchungen als fraglich. Ähnliche Erfahrungen in bezug auf den Einfluss kohlehydratreicher Kost auf die Entwicklung von *Entamoeba histolytica* bei Ratten, machte **Böe** (2). M. E. nach sind die Befunde von **Böe**, **Ghosch** u.a. nicht im Sinne einer für die Amöben günstigen Änderung in der Reaktion des Darminhaltes, sondern als Folge der erhöhten Insulinausschüttung, bedingt durch die kohlehydratreiche Ernährung, zu bewerten.

Die eigenartige Anordnung der Erythrozyten aus defibriniertem Schafsblut an einer Stelle der Amöbenoberfläche in Form eines Bouquets, entspricht wohl mit Bestimmtheit der Polarisierung der *Entamoeba histolytica*. Es dürfte sich nicht um eine beliebige Stelle handeln. Viel eher könnte man sich vorstellen, dass es sich um die Trennungsstelle der Amöbenkörper bei der Teilung handelt. Histologisch könnte es sich hierbei um einen mehr minder gut „abgeheilten" Defekt handeln.

Die ringförmige Anordnung der Erythrozyten, die aus defibriniertem Schafsblut stammen, in gleichmässigen Abstand um die Cysten ist wohl ein Zeichen des Lebens der letzteren. Diese biologische Reaktion, der Enzyklierung auf Distanz, ist so deutlich, hebt die Cysten so gut aus ihrer Umgebung heraus, dass sie zur Stellung der Diagnose verwendet werden könnte.

In Pyocyanin scheint ein Farbstoff gefunden zu sein, der sich möglicherweise auch zu andren, wirklichen Vitalfärbungen eignet. Bei den hier untersuchten Amöben war festzustellen, dass die Amöben eine ein wenig leuchtende grünliche Färbung annehmen. Ferner scheint das Pyocyanin, in geringem Ausmass zugesetzt, auch geeignet, die Beweglichkeit der Amöben zu erhöhen.

Vereinzelte Beobachtungen lassen vermuten, dass eine ektoplasmatische Praeformierung der Exzystationsstelle besteht und dass die Ausbildung und Retraktion eines stielgedrehten Pseudopodiums, den

Zweck hat die später ausschlüpfende Amöbe, mit einer Schutzhülle zu umgeben.

Zusammenfassung.

1. *Entamoeba histolytica* lässt sich auf Liver Infusion Agar durch Zusatz von in Insulin suspendierter Stärke anreichern.
2. Die vegetative Form von *Entamoeba histolytica* ist polarisiert.
3. Die Cysten von *Entamoeba histolytica* zeigen bei Zusatz von defibriniertem Schafsblut, als biologische Reaktion, Enzyklierung auf Distanz. Damit ist eine Erleichterung in der Diagnosenstellung „Cyste" gegeben.
4. Pyocyanin ermöglicht die Vitalfärbung von *Entamoeba histolytica* wobei Struktur und Kern deutlicher sichtbar werden und die Beweglichkeit der Amöben sich erhöht.
5. Vereinzelte Beobachtungen von Stieldrehung eines Pseudopodiums lassen vermuten, dass eine ektoplasmatische Präformierung der Excystationsstelle besteht und dass die Amöben nicht nackt ausschlüpfen.

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(From the Laboratory of Hygiene of the University, Utrecht).

ON THE ACTION OF SULFANILAMIDE

II. THE ACTION OF SULFANILAMIDE ON CATALASE *)

by

H. W. JULIUS AND K. C. WINKLER

(Received February 10, 1941).

The action of the drug sulfanilamide has been subject to a great number of investigations. They have brought to light some striking differences with the action of germicides in general. Two of these are essential for the problem under discussion.

1) The action of the drug, occurring only in growing cultures inoculated with small numbers of bacteria, does not become manifest until after some hours of normal growth („lag time" of action).
2) Addition of blood or serum, so deleterious on the effect of other germicides seems not to decrease sulfanilamide action, but even seems to enhance its effect (1).

As to the mechanism of action of the substance several theories have been proposed (2). Recently an interesting suggestion has been added to the many conjectures by LOCKE, MAIN and MELLON (3), who supposed an inactivation of bacterial catalase by the drug. The resulting accumulation of hydrogen peroxide would be the actual cause of bacteriostasis. On finding the inactivation of catalase by sulfanilamide itself to be very small, these authors supposed the sulfanilamide to be oxidized and the product (amino-sulfon-benzene-hydroxamide) to have inactivating properties (4) in analogy with the anticatalytic action of hydroxamide. For different sulfanilamide-compounds, oxidized by irradiation with ultra-violet light they could record an anticatalytic activity; this property, however, was not correlated with therapeutic activity (4), nor did the action of these

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*) First communication: L. K. WOLFF and H. W. JULIUS, Ann. de l'Inst. Pasteur **62**, 616, 1939.

compounds surpass that of sulfanilamide itself. Amino-sulfon-benzene-hydroxamide, however, seems to act bacteriostatically without a lag time (5).

In cultures containing sulfanilamide MAIN, SHINN and MELLON could note a small increase of hydrogen peroxide concentration per unit of growth (6). Though important, this fact not necessarily proves an inactivation of catalase as a change in bacterial metabolism might as well account for the increased hydrogen peroxide production. The increased Eh of sulfanilamide containing cultures (7) seems to support the theory. However, the Eh of sterile broth with sulfanilamide is increased to about the same extent (8).

Direct evidence for the validity of the theory is thus still lacking; moreover some other objections as to the probability of the conception can be made f.i.

1) The oxidation of sulfanilamide into an anti-catalase is pure conjecture. We believe, we are able to show that sulfanilamide is not converted before action (9).
2) The bacteriostatic action of sulfanilamide on streptococci is much enhanced by the addition of blood, a rich source of catalase. An inverse effect might be expected, if sulfanilamide were to act by inactivation of catalase. The action of sulfanilamide *in vivo* on streptococcaemia would imply an inactivation of the greater part of the blood catalase since an (local) accumulation of hydrogen peroxide just around the coccus (MAIN, LOCKE and SHINN l.c. Chart I) by local catalase inactivation is inconceavable in streaming blood.
3) Some strains of streptococci do not produce hydrogen peroxide at all, but still are sulfanilamide-sensitive (10).
4) Anaerobiosis excludes peroxide formation and sulfanilamide should not affect anaerobic growth. SHINN, MAIN and MELLON indeed report a reduced activity of sulfanilamide under low oxygen tensions. At very low oxygen tension ($< 0.04\%$), however, they find sulfanilamide active again (11). They suppose another mechanism of sulfanilamide action in this case.

   A bacteriostatic action of sulfanilamide on anaerobes has been clearly shown, however, for *Clostridium sporogenes* (12). Furthermore streptococci, cultivated anaerobically are not always resistant to sulfanilamide and the resistance has been shown not to be due to anaerobiosis per se but rather to properties of the used strains (13).

We did not find any difference in bacteriostatic action of sulfanilamide on *Streptococcus haemolyticus* or *Bacterium coli* cultured aerobically and anaerobically (14).

To try to obtain some direct evidence about the action of sulfanilamide on bacterial catalase we made use of the very ingenious method employed by JOHNSON and VAN SCHOUWENBURG (15) to demonstrate traces of oxygen by means of luminous bacteria.

Method.

Fig. 1 shows the instrument of JOHNSON and VAN SCHOUWENBURG. *C* contains a culture of *Photobacterium Fischeri*, *B* the substance to be tested for catalase and *A* a 0.1% solution of hydrogen peroxide in saline buffered at pH 7.3. Connections are made with leaden tubes and de Khotinsky cement (for details see the cited paper). Oxygen-free hydrogen is bubbled through the system until the luminous bacteria in *C* are no longer visible (dark room, sufficient adaptation!).

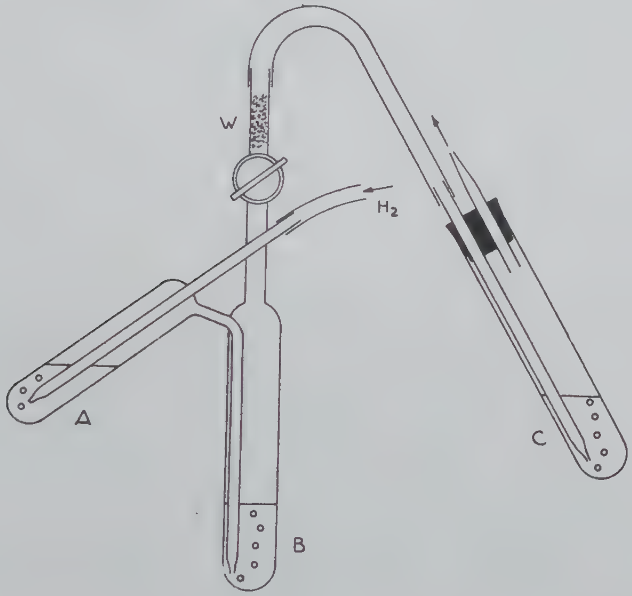

Fig 1.

By tilting the system ABC the peroxide is added to the substance in B. If catalase is present oxygen will be produced and after some time (one to three minutes) the luminous bacteria in C will regain their luminescence.

If B contains blood or serum a cotton plug with a drop of amyl-alcohol had to be placed at W (fig. 1) to prevent foam bubbles to reach C. With blood in C it was practically impossible (only after hours) to obtain complete darkness in C, no difficulty however was felt in observing the effect, since the luminescence produced by the freed O_2 was very marked in those cases.

We found it possible to record the luminescence effect by fastening a piece of Agfa Isopan ultra film (23/10 Din) to the tube C during an exposing time of 10 minutes. A strip of black paper luted to the tube furnished a non exposed mark.

Results.

I. Demonstration of bacterial catalase.

By filling B with a culture of

a) *Bacterium coli* in plain broth,

b) *Photobacterium Fischeri* in 1% peptone with 0.1% glycerol and 3% NaCl,

c) *Streptococcus haemolyticus*, strain Aronson, in infusion broth with 10% horse serum,

the return of luminescence in C after tilting was very clear, which demonstrates the occurrence of catalase in cultures of these organisms. Controls with the used media and with boiled cultures proved to be entirely negative, thus demonstrating, that there was no unspecific effect. The effect was marked in the cases a and b, and less so, but still quite distinct with the streptococcus. So this strain of *Streptococcus haemolyticus* produces catalase; this is interesting as in the view of some authors streptococci in general do not (16).

B. coli, Ph. Fischeri and the used strain of Streptococcus. haemolyticus produce catalase.

II. Does sulfanilamide inactivate blood catalase?

To 2 ml of sterile sheep-blood + 6 ml of plain broth 2 ml were added either of a 0.5% solution of sulfanilamide in aq. dest., or of a 0.1% and 1% solution of hydroxamide; 2 ml of aq. dest. served as a control. Furthermore a mixture was prepared with 2 ml of a 0.5% solution of sulfanilamide, which had been irradiated in a 0.5 cm layer for two minutes with a Philips high pressure mercury-lamp HP 75 at 10 cm distance.

The luminescence induced with mixtures containing sulfanilamide, was not visibly different from the effect induced by the control. So we failed to demonstrate an inactivation of catalase by sulfanilamide. With the irradiated compound a small decrease in luminescence could be recorded photographically.

With hydroxamide in aequimolecular concentration the intensity of the produced luminescence was somewhat less, but the inactivation was far from being complete. To obtain this a ten times greater concentration, *i.e.*, a final concentration of 0.2% was needed.

To inactivate blood catalase *in vivo* the sulfanilamide or its oxidation products would have to be about 500 times as active as hydroxamide!

Of course the theory of MELLON, LOCKE and SHINN supposes local inactivation of catalase around the cocci, but this is at variance with the pronounced action of sulfanilamide *in vitro* in well stirred 50% blood broth media. Furthermore not even a partial inactivation of catalase could be noted in a 24 h. culture of streptococci on this medium containing sulfanilamide.

Sulfanilamide 0.1% does not inactivate the catalase of a 20% suspension of sheep-blood in plain broth.

III. DOES SULFANILAMIDE INACTIVATE BACTERIAL CATALASE?

To 8 ml of a 24 h. culture of *Ph. Fischeri*, *B. coli* and *Streptococcus haemolyticus* on their respective media were added 2 ml of a 0.5% solution of sulfanilamide (final conc. 0.1%) or aq. dest. as a control. Then they were tested in the apparatus described above.

No difference in luminescence of the luminous bacteria in C (fig. 1) could be observed between the sulfanilamide containing cultures and the controls either when the experiment was carried out immediately after the addition of sulfanilamide, or when the sulfanilamide had been left in contact with the cultures for five hours before the peroxide was added. So sulfanilamide does not inactivate bacterial catalase.

Still the supposition might be made that sulfanilamide might either act merely on (or in) growing bacteria or on the production of catalase. In growing however *Streptococcus haemolyticus* on a 10% horse serum broth (catalase free) with a final concentration of 0.05% sulfanilamide for five hours (that is just about the period that bacterial growth in both media is still equally rich), no difference could be observed with the control culture without sulfanilamide.

After 24 h. the absolute catalase activity of the control is much greater than that of the sulfanilamide containing culture, but this is clearly due to the much greater number of bacteria in the former case. If the action of sulfanilamide is due to inactivation of bacterial catalase, its effect should occur after five hours, for this is just about the time after which bacteriostasis begins to show in the growth curve whilst the number of bacteria is still about equal in both cultures (1).

For *B. coli* this could be demonstrated as well.

In similar experiments with *Streptococcus haemolyticus* growing on a catalase containing medium (50% blood infusion broth; a growth curve was determined simultaneously), no difference in catalase activity occurred between sulfanilamide containing cultures and controls after 5 or 24 hours, as was to be expected in view of the results described in II.

Sulfanilamide does not inactivate bacterial catalase nor does it affect the production of catalase in the growing culture containing sulfanilamide.

Summary.

A. According to Mellon, Locke and Shinn, the bacteriostatic action of sulfanilamide is due to the inactivation of (bacterial) catalase and the resulting accumulation of hydrogen peroxide. The probability of this theory is discussed.

B. Catalase activity was studied by means of *Photobacterium Fischeri*, as an oxygen indicator. By adding hydrogen peroxide to the tested cultures of bacteria it has been demonstrated, that:

I. *Bacterium coli*, *Photobacterium Fischeri* and *Streptococcus haemolyticus* (strain Aronson) contain catalase.
II. Sulfanilamide does not inactivate the catalase in blood.
III. Sulfanilamide does not inactivate bacterial catalase nor does it affect the production of catalase in the growing culture containing the drug.

So we have to conclude that the assumption of catalase inactivation to be the essential factor in sulfanilamide action on bacteria will not lead us to the solution of the problem.

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(From the Laboratory for Hygiene, Bacteriology and Tropical Hygiene of the University, Leiden).

ON A BACTERIUM THAT, WHILE RETAINING ITS FULL BIOCHEMICAL AND SEROLOGICAL PROPERTIES DEVELOPS ON THE AGAR PLATE IN TWO COLONY FORMS

by

S. J. C. DUNLOP

(Received March 3, 1941).

In May 1939 I isolated from the urine of a female patient in the Haven Hospital at Rotterdam, a bacterium showing some properties which are so remarkable that a more detailed description seems justified. In existing literature I could not find any mention of similar phenomena, but it is, of course, possible that the phenomenon has already been ascertained by somebody else.

The patient in question was a woman of 54, with a prominent ptosis of all abdominal organs. Probably this had led to the pinching off of the bladder neck causing stagnation of the urine. In any case the cathetised urine was found to be extremely turbid, containing the bacterium as it were in pure culture. On repeating the examination twice, I met both times with the same bacterium in pure culture.

Yet it is unlikely that this is a case of actual cystitis, as the urine, after treatment of the general ptosis, which resulted in its disappearance became clear along with the disappearance of the bacteria from it. At the beginning the urine contained a rather high percentage of protein and a trace of sugar. Both disappeared after treatment of the ptosis. The bacteria it is true, agglutinated with the patient's own serum to a titre of 1 : 1600, but this does occur in cases of saprophytes that have been in contact with the human body for a long period and have got into the vascular system. So this should not be considered a symptom of pathogenity. It is strange, however, that the urine contained the bacterium in pure culture and that no coli bacteria occurred.

This introduction brings me to my proper subject, the isolated microorganism. This is a gram negative coccus ca 0.8 μ in diameter. It is non-motile and occurs as separate cocci, so not in diplo-, strepto- or staphyloform. As far as I ascertained them, its biochemical properties are the following:

Acid is formed in maltose, mannitol, lactose and glucose, not in saccharose. No gas in lactose and glucose. Indol is formed in 1—2 × 24 hours. Litmuswhey stains red. In broth the growth is slightly turbid with a rather pronounced sediment. Gelatine is not liquefied and haemolysis develops on the bloodagar plate and likewise in the blood broth.

The behaviour of this bacterium on the agar plate however was peculiar. At the time of isolation, I was directly struck by the fact that there occurred two different colony forms on the plate. One of these was a rather large turbid colony with a clear zone along the edge and a smooth surface; the other type was a minute colony, only by means of a pocket lens well visible, and also smooth and shining but clear.

My first thought was a mixed infection. However, on trying at separation, the following remarkable fact was disclosed: When a large colony had been streaked on a new plate, only large-sized colonies developed. On the other hand, when a small-sized colony had been transferred under a pocket lens with a thin platinum needle and been streaked on a new plate, both large and small colonies could be observed (cf. fig. 1); initially a few large colonies against a good many small ones, but on keeping the plate without opening it at a temperature of 37° C. for some more days, large colonies kept on developing in increasing numbers. To this day I have not succeeded in obtaining a pure culture of small colonies. Not all small colonies develop into large ones however; by far the majority remain small.

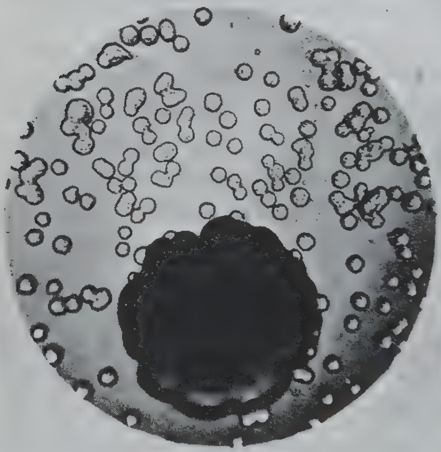

Fig. 1. One large colony and small colonies on an agar plate, after one day at 37° C 20×.

In order to exclude the possibility of a mixed infection, I set to work as follows: as a first method I kept a plate at a temperature of 37° C. during three weeks; subsequently I transferred a colony, that had remained small-sized, under a pocket lens to a new plate, repeating this process ten times. The tenth transfer still resulted in the occurrence of both large and small colonies.

As another method of investigation I made six one-cell cultures from a 24 hours small colony. From these five developed, all of them producing in the same way large and small colonies.

I also tried to obtain one-cell cultures from a small colony aged three weeks, but none of the twelve cells isolated would develop. Probably the chance of isolating a living germ from such an old culture — the bacterium moreover being non-motile — is so slight, that for obtaining a positive result a considerable number of isolations would have to be effected. The time needed for this failed me, however, and such a test seemed moreover superfluous.

In order to offer some figures, the average diameter of 30 large and small colonies at various stages of their development will be recorded.

	The small colonies	The large colonies
after 24 hrs	0.11 mm	—
,, 48 ,,	0.29 mm	0.89 — 2.87 mm
,, 10 days	0.89 mm	2 — 4 mm
,, 22 ,,	1.06 mm	5 mm

After some three weeks the colonies have reached their maximum size. The large colonies clearly give the impression that they have developed from the small ones. So a small colony with a dark spot

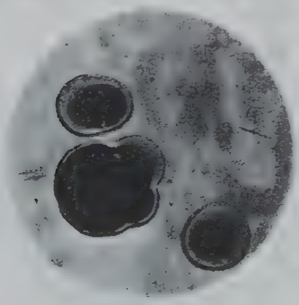

Fig. 2: Development of a large colony from a small colony, after two days. 20 ×.

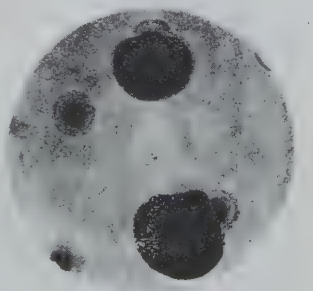

Fig. 3. Development of a large colony from a small colony, after two days. 20 ×.

somewhere at the edge or in the centre may be ascertained (cf. fig. 2). This spot develops into an outgrowth which grows around the small colony; this growing colony becomes kidney-shaped with the remains of the small colony in the inner curve (cf. fig. 3). Ultimately the small colony entirely disappears and a completely round large colony is formed.

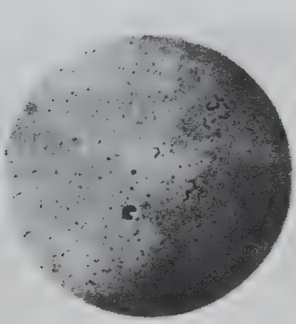

Fig. 4. Small colony after one day.

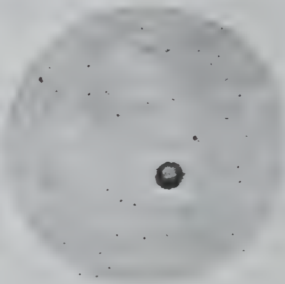

Fig. 5. Small colony after two days.

To make a series of photographs of a small colony developing into a large one proved to be very difficult and expensive, as it is impossible to tell beforehand which colony will increase in size. The colonies that remain small degenerate in the long run, like every colony on agar, into a granular mass (cf. figs. 4—8, all 20×).

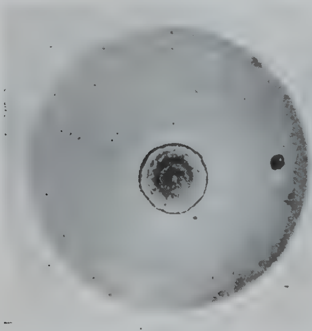

Fig. 6. Small colony after one week.

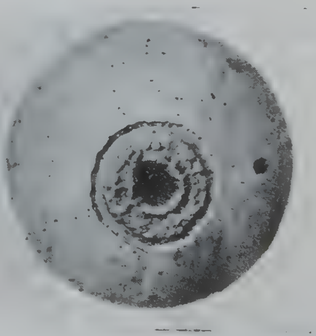

Fig. 7. Small colony after two weeks.

I further tried to demonstrate the serological relationship of the microbes from the different colony forms: 1. colonies that had been large-sized since the first isolation. 2. colonies developed from small into large ones and 3. small colonies. The latter were the hardest to obtain in pure culture. They were obtained by streaking a small colony on a plate. The few large colonies that had developed were cut out with the surrounding agar and the remaining colonies were suspended in 0.85% NaCl. At the end of the immunisation I initially effected a cross-agglutination with the following sera:

Serum of a rabit immunised with colony type 1: serum 1.
Serum of a rabit immunised with colony type 2: serum 2.
Serum of a rabit immunised with colony type 3: serum 3.

The results are the following:

	Serum 1	Serum 2	Serum 3
Large colony	1 : 400	1 : 6400	1 : 6400
Colony developed into a large one	1 : 400	1 : 12800	1 : 3200
Small colony	1 : 400	1 : 12800	1 : 6400

Subsequently serum 3 was saturated with the three different colonies, in accordance with CASTELLANI's diagram, and finally tested on possible remaining agglutinines. The result was that in all nine combinations the agglutinines were entirely adsorbed, if the test had been executed with sufficient accuracy. Saturation with the small colonies proved to be the most difficult, as it was anything but easy to obtain a sufficiently thick suspension for a full saturation. Moreover, I effected a complement-fixation reaction with the three different antigenes. All nine possible combinations showed a complete complement-fixation.

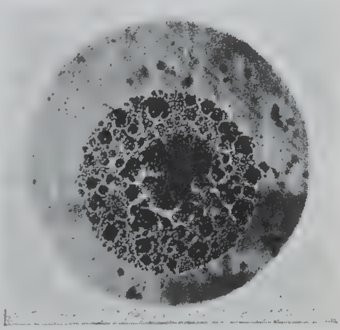

Fig. 8. Small colony after three weeks.

So I could not find any marked difference between the three forms of colonies.

Discussion and conclusion.

The question now remains how the above must be accounted for and what conclusion should be drawn. Is this a case of a S-R variation? With the exception of the fact that the large form does not change back into a small one, all facts tend towards the contrary. The large colony-form would represent the R-form. However, there is no difference in antigenic structure, no difference in hydrophility and the large colony is not Rough but Smooth. The virulence cannot be studied, as the bacterium is not a pathogenic one. Furthermore, to my knowledge, such a pronounced difference in size of a S-R variety has never yet been described.

In my opinion we have here come across a continually occurring mutation, variation, bacterial dissociation, or whatever it might be named. The suggestion then presents itself that this is a case of a bacterium which, under what seem to us normal conditions, is capable of continually occurring under two forms, one of which only is germfirm. I would suggest that the large form is the normal one, which might be expected to appear on the agar, were it not that, owing to a special influence, as yet unknown to me, a great number of individual cells develop into small colonies.

Once a certain germ has outgrown this unknown influence, it is capable of developing a large colony, which, in fact, is the stable form.

Of what nature this unknown influence is I have been unable to determine so far. I investigated whether one of the types might be lysoresistent and the other lysosusceptible. It was easy enough to find a bacteriophage which dissolved either form, but both proved to be lysosensitive and to retain their original properties even after treatment with the phage.

So up to the present I cannot offer any opinion as to the cause of this peculiar phenomenon.

Summary.

A bacterium has been described which had the property of continually developing on the agar plate in two colony forms, that had the same biochemical and serological properties and did not show any morphological difference either. The cause of this variety in development is left unexplained.

(From the Laboratory for Hygiene, Bacteriology and Tropical Hygiene
of the University, Leiden).

ARE BACTERIOPHAGES SIGNIFICANT FOR THE SELF-PURIFICATION OF SURFACE WATER?

by

P. C. FLU

(Received March 3, 1941).

All surface water and more especially that which occurs in the neighbourhood of human dwellings or passing between them is open to pollution. The polluting elements consist of inorganic and organic matter in solution and of corpuscular elements, varying from the smallest organisms to all kind of detribitus and coarser refuse of man and animal, domestic garbage, next to unsoluble inorganic substance such as sand, coal, etc.

Since long it is a well-known fact, that stagnant as well as streaming surface water has in a high degree the faculty to purify itself from the pollution it is subject to. Many factors play their part in this so-called ,,self-purification". First of all the dilution; solved substances especially are reduced in this way to minimum concentrations. Secondly the mechanical reduction and division which result from the disjoining action of the water, from the motion of its waves as caused by the wind or by the stream. Sedimentation is of major importance; it induces clarity of the water. At the bottom of the water reservoir a layer of organic and inorganic matter of varying depth settles, which may get putrified and which in case of an extensive and constant pollution may render the water less and less adapted to any purifying action. The sunlight has also a considerable influence on the occurrence of the purification. This influence is of such importance, that by cutting off the direct or indirect sunlight, the purification process may be greatly slowed down and may even be brought to a standstill.

However important these physical factors may be, none of them either separately or combined would bring about the purification, were they not followed or supported by biological processes, connected with the nutrition and assimilation of lower and higher plants and animals. Although the degree of dilution of solved inorganic and partly of organic matter as well would in the long run increase in stagnant water to a high concentration, were it not that this matter were taken up for assimilation either by bacteria or more frequently by fungi and higher plants and animals. Bacteria bring about the putrefaction of the organic matter, which in this way is transformed into substances with lower molecular weight; the latter will ultimately be reduced to inorganic compounds such as NH_3 and CH_4. These will either evaporate or be transformed into nitrites and nitrates. Aquatic animals, protozoa as well as lower and higher metazoa play a major part in the removal of submicroscopic and microscopic suspensa and even of such of macroscopical dimensions.

Among the microscopic suspensa occurring in polluted surface water, chiefly the bacteria and among them more especially the pathogens awake the interest of the epidemiologist and the hygienist.

For nearly all known pathogenic germs water, either pure or the more so when heavily polluted, is not the most suitable medium. When pathogenic germs, even those causing water born diseases, occur in water, they will vanish after a more or less extended lapse of time, depending on the species of the organism. As this fact has been substantiated by nearly all investigators, I need not go into further detail. Which are the factors which coöperate in the removal of the pathogenic germs in the water?

In 1904 EMMERICH and GEMUND pointed out the significance of flagellates for the self-purification of water, which according to the authors was induced by these animals (5). Later on it has been pointed out that not only flagellates act as purifiers. The part of other groups, *viz.*, amoeba and especially ciliates is even more prominent. The amoeba with their pseudopodia and chiefly the ciliates with their cytostomata capture great numbers of organisms. In a medium containing ciliates and bacteria it may be observed under the microscope that these protozoa by means of cilia, cirri and membranellae maintain a current which tends to the cytostoma. A variety of suspensa are carried along with the current. The coarser ones are held back by the barrier of cilia, cirri or membranellae, but the smaller ones *e.g.* the bacteria are carried into the stoma and cytopharynx. Later they may be observed in the food vacuole where they will be quickly digested.

According to many scientists, pathogenic germs would exert a stimulating action on aquatic protozoa. The multiplication of the latter would be accelerated and in this way a more rapid decrease in bacterial numbers would be induced.

KRUSE (13) is of the opinion that water bacteria will act antagonastically on bacteria which do not have water as their normal habitat. He arrived at this surmise, since according to him not the protozoa merely could be made responsible for the vanishing of the germs from the water.

STOKVIS and SWELLENGREBEL (20) recorded that *Colpoda cucullus* and *Bodo ovatus* were able to clarify turbid suspensions of typhoid bacteria and cholera vibrios completely in a few days. At temperatures lower than 6°C. or above 37°C. no clarification occurred. Extracts of protozoa did not influence the clarification, nor could any antagonistic action of bacteria be recorded. Merely living *Colpoda* could induce the purification.

At an earlier date already STOKVIS (19) had found, that the vanishing of organisms from water could be interfered with by the addition of 50 mg KCN per liter. According to him KCN in this concentration does not act on the bacteria; protozoa in the vegetative stage, however, are killed off and their development from cysts is inhibited.

KISSKALT (12) recorded that the supply of KCN in the amount of $1^0/_{00}$ to water which is filtered through slow filters, interferes with the filtration process; protozoa are killed, but bacteria are not.

MÜLLER (14) made another important contribution; he observed that besides KCN saponine, a poison for protozoa, interferes with the purification and that after contamination of an open water the number of organisms increases initially. Later on the number of protozoa increases what is followed by a decrease in bacterial numbers.

The results of an examination into the cause of the vanishing of cholera vibrios which had been supplied in smaller and greater numbers to water of Netherlands Indian rivers kept in 40 liter tanks, led me to the conclusion. that the action of protozoa preponderated. Although in boiled water from the Tjiliwoeng and Kroetoet rivers [1]) cholera vibrios may be kept living during several weeks, they will vanish in 3 to 8 days in untreated water from these rivers when kept in tanks; this will occur as well when large amounts of bacteria (the entire mass of bacteria from the surface of an agar plate of 18 cm diameter) have served as inoculum. When the water had been contaminated with the feces of cholera patients such rapid disappearance could be observed as well (6).

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[1]) The Tjiliwoeng and Kroetoet rivers stream through densely populated quarters of Batavia.

In a later paper I claimed that the artificial conditions, thus as they are in use in the laboratory for the investigation of the self-purification of water, correspond in a way with the conditions prevailing in stagnant pools (7). In the water of streaming rivers protozoa may also be a factor, but they are of minor significance when compared with the preponderating factor, *viz.*, the very high dilution of the contaminant.

Shortly after the discovery of the bacteriophage it has already been tried to connect phage with the disappearance of germs from surface water. According to D'HÉRELLE (11), HANKIN would have been the first to note a phenomenon, which in D'HÉRELLE's opinion is to be ascribed to the action of the bacteriophage. HANKIN (10) had pointed out, that water of the river Jumna apparently destroyed the cholera vibrios occurring in it. The examination of water from this river for cholera vibrios gave none but negative results, notwithstanding a heavy contamination with feces. The results were still in the negative, when water samples had been gathered in a region which surely had been contaminated, *viz.*, in the direct neighbourhood of corpses of cholera patients floating on the water.

As early as 1926 (8) I have claimed that after a study of HANKIN's paper one cannot possibly agree with the statement of D'HÉRELLE. As it is HANKIN lays stress on the fact that the substance which is responsible for the vanishing or the cholera vibrios is of volatile nature. When water of the Jumna had been heated in a sealed tube in the autoclave at 115°C., it had not lost its bactericidal action. This however had vanished when the tube had been kept open during heating. As a bacteriophage will be destroyed with certainty at a temperature above 70°C., it is out of the question that the action of bacteriophage might explain the phenomenon recorded by D'HÉRELLE.

Dating from 1923 a series of papers have been published, which deal with the problem of the self-purification of surface water.

ARLOING and SEMPÉ (1) have in a series of papers discussed the part which the bacteriophage might play in this purification. They state that not all surface waters will contain bacteriophage. Water from a same geographical area is generally equally lytic. A lytic action occurs mainly in slowly streaming rivers and the more so if they are strongly polluted. This could be recorded for the Seine, Saône and Deule. The sea water on the high sea has no lytic action. A similar investigation was extended over rivers in Europe and in the Old and New World. They deem it probable that phage is a factor in the self-purification of polluted water. In an examination of the river Deule which passes through Lille, BILOUET (3) detected very virulent phages for intestinal organisms. The concentration of phage increased after heavy rainfall along with a dilution and a heavy contamination of the river water. He records the largest content of phages in the river above Lille and very varying numbers below the city. According to him this distribution does furnish an argument in favor of the phage as a factor in the purification of the river Deule. In the lagoons of Venice TATUNATO could always detect phages; in a distance of 2 km from the harbour of Messina they occurred as well. The phages traced by him were always for *B. coli* and *B. dysenteriae* Shiga, but never for typhoid- or paratyphoid bacteria. In his opinion this might be due to the purifying action of the phage. No direct proof, however, for the hypothesis is furnished.

PANAYAOTATON (15) points out that the river Nile as far as it has been polluted by the neighbourhood of men is rich in active bacteriophages; without pollution no phages occur.

STEWART and GHOSAL (18) arrive at the conclusion that bacteriophages do not influence the purification of water in sand filters. Sand-filtration distinctly destroys phages, which occur in the non-filtered water. Sedimentation of the water before the filtration results in a decrease in numbers of phages of 90—99%.

BECKWITH and ROSE (2) who could always detect phages in sewage, state that the content of phages herein varies according to the season and that it increases after heavy rainfall. Many but not all phages in sewage are resistent to chlorination.

Although the conditions in sewage may not be optimal for increase and adaptation it seems to me that phages may not only be able to impede the growth of microbes but may as well induce in some degree purification of the fluid as to intestinal organisms. BUJANOWSKI (4) among others tried to furnish direct proof of the part which bacteriophages might take in the purification of water. He inoculated water from the river Don which had been filtered through Chamberland candles L3 with pathogenic bacteria and studied the behaviour of these bacteria by means of a comparison with their growth in water sterilised by boiling before inoculation. In filtered Don water the bacteria succumbed after 2 or 3 weeks, whilst in sterilised water they remained living up to six months. Apparently he ascribes the vanishing of the germs in the filtered water to the action of phages, without accounting for the fact, that the phage will act after 24 hours and not after 2 or 3 weeks.

In 1926 I studied the self-purification of surface water which was kept in the laboratory; this investigation was meant to test the claim of D'HÉRELLE, that bacteriophage would be a preponderate factor in this purification. I could state that Shiga- and typhoid bacteria vanished at the same rate from water, which contained none but phage for Shiga bacteria. No increase in the titre of phage could be recorded in water out of which Shiga bacteria had vanished. When typhoid bacteria and cholera vibrios had vanished no phage for either organism could be detected, either before or after the disappearance, even when samples of 25 ml were put to the test. The results of this investigation made me conclude, that the significance of bacteriophage in processes of self-purification is doubtlessly small. No proof whatever could be furnished of any part played by the bacteriophage. The purification of water may occur in the complete absence of phage, whilst its presence does not accelerate the process. The experiments furnished another proof of the important part protozoa take in the purification. When under laboratory conditions I eliminated the protozoa, the self-purification was inhibited, even when the water had been provided with bacteriophage.

In a series of papers SCHUURMAN and SCHUURMAN—TEN BOKKEL HUININK (17, 18) have published the results of their investigations on the occurrence of phages for typhoid bacteria in the river Tjiliwoeng in Java and on the part played by these phages in the self-purification of this river. They pointed out that these typhoid phages parasitise on aquatic bacteria. These phages and the latter bacteria are connected with each other and next to this activity they develop an activity against typhoid bacteria. Although these investigators could not by direct observation record any vanishing of typhoid bacteria in untreated water, they admit its occurrence, because they could detect an increase of typhoid phages after an inoculation with typhoid bacteria.

It seems to me that the results of my own investigations allow me to claim that water is not a normal medium either for pathogenic bacteria or for phages (6).

The further question arose whether phages might vanish during the self-purification, such as it had been proven for the intestinal organisms. The phage for Shiga bacteria seemed to me most suitable to answer this question. It occurs in each surface water, whenever this has been contaminated, however slightly, with feces of man or animal. The concentration of the phage in such water may be high and it may be traced in dilutions as low as 10^{-2} to 10^{-5}.

6 l water from the „Doelengracht" was poured into a bottle of white glass; a rubber stopper prevented contamination during the investigation and the bottle was kept in diffuse daylight at room temperature (18°C.). The self-purification in the thus conserved water was examined by determination of the coli titre according to EIJKMAN and of the titre of the Shiga phage. To this end several samples of the water were mixed with broth and inoculated with Shiga bacteria. After incubation during 24 hours at 37°C. and filtration through Chamberland candle L3 the filtrate was examined for phage. The results are taken up in Table 1.

Table 1.

Date	Coli titre according to EIJKMAN	Phage titre for Shiga bacteria
13/3 [1]	10^{-3} ml +	10^{-3} ml + 10^{-4} —
28/3	0.5 ml +	10^{-3} ml + 10^{-4} —
9/4	of (5 × 1 ml) 1 ml +	0.5 ml +
19/4	of (6 × 1 ml) all —	of (3 × 0.1 ml) all —
23/4	of (17 × 5 ml) 1 × 5 ml +	of (10 × 1 ml) 1 ml —
16/5	250 ml —	of (10 × 1 ml) all —

The results indicate that canal water is not a suitable medium for the multiplication of the Shiga phage; it induces a vanishing of the phage. The disappearance of the coli bacteria is to be ascribed for one part to protozoa which will feed upon them, for an other part to the low nutritional value of the medium, which will cause a dying off for lack of food. The causes of the vanishing of the phage are probably manifold. The low content of protein in the medium is certainly a factor. Moreover phages will disappear in water which is not buffered by the interaction of uni- and bivalent cations on a well suitable level for the phage. It has been pointed out by me (6) that bacteriophage for *Bac. megatherium* after an interval of a few days is inactivated in distilled water. Weak salt solutions of uni-, bi- or trivalent cations are very harmful. The injurious action of univalent cations can be counteracted by a suitable, usually very slight, amount of bivalent cations.

The results of this investigation, which are not in harmony with the conclusions of several investigators who claim that phages are an important factor in the self-purification of water, made me wish to obtain by means of some quantitative experiments a further test of my claim about the low significance of phages in the self-purification.

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[1] Beginning of the investigation.

I have already mentioned the work of SCHUURMAN on typhoid phages in the water of the Tjiliwoeng. He claims that the high content of typhoid phages in the Tjiliwoeng does not point to the occurrence of numerous typhoid bacteria, but may rather be due to the lysis of other species of bacteria, susceptible for the phage. In non-filtered water of the Tjiliwoeng he cannot trace any increase in number of the typhoid bacteria, in heated water of the Tjiliwoeng and in heated filtrate, however, increase occurs. In non-heated L3 filtrate of Tjiliwoeng water typhoid bacteria vanish. It might hardly be expected otherwise: if typhoid bacteria will multiply in heated filtrate, they have to vanish if this filtrate contains the typhoid phage. SCHUURMAN himself perceives that either the fact, that typhoid bacteria may multiply in heated filtrate of Tjiliwoeng water, nor the fact, that this keeps true for heated non-filtered water, nor finally the fact that typhoid bacteria are lysed in filtrate of Tjiliwoeng water containing typhoid bacteria, may furnish proof of the typhoid phage playing a part in the purification under natural conditions. Although he could not record any increase of typhoid bacteria in non-filtered water, he still claims such an increase to exist, because of the fact, that the titre of typhoid phage increases in Tjiliwoeng water, when this has been contaminated with typhoid bacteria. In his opinion an increase in the phage titre might not be deemed possible without a multiplication of the organisms at the cost of which the phage increased. To substantiate his claim SCHUURMAN (16) inoculates flasks, which have been provided with 100 ml non-filtered Tjiliwoeng water, with typhoid bacteria and records an increase of the phage titre from 10^{-1} to 10^{-2}. An equal increase is reached, when non-filtered Tjiliwoeng water has been inoculated with water bacteria from the Tjiliwoeng, which are lysable by typhoid phage. He records moreover, that non-filtered Tjiliwoeng water which has been inoculated with typhoid bacteria, is after a lapse of 24 hours at 28°C. slightly clarified, when compared with an equal amount of heated Tjiliwoeng water, which has been inoculated with a same amount of typhoid bacteria. From these results he draws the following conclusions: the latter titration experiments have furnished proof that typhoid phages are an important factor in the self-purification of Tjiliwoeng water against typhoid bacteria and homologous phages are active against its natural bacterial population; the hypothesis that the bacteriophage is a factor in the maintaining of a biological balance in nature has been confirmed by a direct proof. One can hardly agree with these conclusions. SCHUURMAN has omitted in his laboratory experiments to eliminate the part which protozoa may play in the self-purification under the prevailing conditions. He merely records that typhoid bacteria in a medium containing typhoid phages are lysed. This fact, however, has no connection with the part taken eventually by phages in self-purification. The fact that the phage titre increased slightly after the inoculation of Tjiliwoeng water with typhoid bacteria may have been the cause of the lysis. It is however not necessarily so. As it is SCHUURMAN points out that along with an increase in the number of bacteria occurring normally in Tjiliwoeng water, equally an increase in the titre of typhoid phage can be recorded. When Tjiliwoeng water has been supplied with typhoid bacteria, however, the normally occurring bacteria will increase in number as well and phage will be produced. So it seems impossible to solve the problem by following the line of SCHUURMAN's experiments. Therefore I decided to repeat my experiments such as they had been carried out in 1926, now however taking the quantitative side in consideration.

First of all I shall describe the technique followed.

The estimation of the number of protozoa.

250 ml of water are filtered through paper until 1—2 ml are left on the filter. During filtration part of the liquid is drawn up from the filter into a pipette and with this portion the material adhering to the paper is washed down into the water which is still to be filtered. In this way all suspensa get gathered in a small volume of water. As soon as this volume amounts to ca 2 ml it is drawn up and its volume is exactly measured. 0.05 ml is transferred to a slide and covered with a coverslip of 22 × 22 mm. The Zeiss cardioidcondensor is used in the counting of the protozoa. The stative is especially built for the condensor and is outfitted with the Zeiss objective C and a compensation ocular 20 ×. The tubus is not drawn out. With this adjustment the diameter of the visual field, measured with an object micrometer, proved to be 0.14 mm. So its area was 0.015 mm^2, or 1/32266 of the area covered by the coverslip.

By counting the number of protozoa in a series of adjacent fields and by dividing the total number of protozoa by the number of fields examined, an average is arrived at. This figure is multiplied by 32266 × 20 and the product is divided by a factor, arrived at by dividing 250 by the volume in ml of the liquid left on the filter. In this way the number of protozoa in 1 ml is obtained:

$$\text{Number of protozoa in 1 ml} = \frac{\text{number counted}}{\text{number of fields}} \times 32266 \times 20 : \frac{250}{\text{n(ml)}}.$$

Estimation of the number of bacteria.

Dilutions of 10^{-1} to 10^{-5} are mixed with the melted agar, cooled down to 45°C. and solidified in petri dishes. After 3 days at 37°C. the number of colonies is counted on these plates as far as the colonies formed are sufficiently separated.

As it had soon turned out, that the plate count of the bacteria decreased in the control bottles with KCN, although the turbidity remained the same, the bacterial numbers of these bottles have been directly counted by means of the counting chamber of Petroff—Hausser. This could only be realized, when the number per ml surpassed a certain limit. When the number was below 10^6 per ml, no bacteria could be detected in the squares of the counting chamber. In such a case a rapid evaporation of most of the liquid under the bell jar of an air pump might have led to results.

Estimation of the phage titre.

This titre was estimated by mixing 1 ml of the dilutions, used for plate counting, with broth, a further mixing with young cultures of the bacteria, the phage against which was to be detected, and by keeping this mixture at 37°C. for 18 hours. After filtration the phage was estimated in the filtrate.

EXPERIMENTS WITH SHIGA BACTERIA.

In every experiment two volumes of ca 5 liters were examined, each taken up in a bottle of 8 liter capacity which was closed with a well fitting stopper. To the contents of every second bottle 50 mg of KCN per l were added. The KCN was chemically pure and was always kept in containers sealed by means of heat.

Either bottle is provided with 5 liters of Rhine water („Galgenwater"). One of them is provided with 50 ml of KCN, whilst both are inoculated with the entire culture of Shiga bacteria grown on an agar slant during 18 hours, suspended in 4 ml of 0.9% NaCl. Previously the number of protozoa had been determined; it amounted to 954 per ml. The phage titre against Shiga bacteria was 10^{-4}. Directly after the supply of KCN and Shiga culture the number of bacteria was determined by plate count (Table 2).

Table 2.

Date	With KCN			Without KCN		
	Number of protozoa per ml	Number of bacteria per ml	Phage titre	Number of protozoa per ml	Number of bacteria per ml	Phage titre
17/6	954	1.650.000	10^{-4}	954	1.780.000	10^{-4}
18/6	0	790.000		4.032	70.000	
19/6	0	397.000	10^{-3}	4.646	1.000	10^{-3}
20/6	0	6.000		2.501	700	
22/6	0	100.000	10^{-3}	1.547	1.140	10^{-1}

This preliminary experiment indicates a decrease in number of viable bacteria in either bottle, although this reduction is much stronger in the liquid without KCN. We were under the impression that although bacteria in the bottle with KCN may die off, no removal as the result of the capturing by protozoa occurs, as the latter are absent. A further experiment in which the number of bacteria in the water containing KCN would be determined by direct counting next to the plate counting, might furnish a test, whether this impression was well founded.

On June 24th the experiment was started. Once again water in two bottles was kept in diffuse daylight at room temperature (22°C.), one provided with 50 mg KCN per liter, the other left without. The day before rain had fallen after a long spell of dry weather. The results are recorded in table 3 and fig. 1.

Table 3.

Date	With KCN				Without KCN		
	Number of protozoa per ml	Number of bacteria per ml counted by PETROFF—HAUSSER app.	Number of bacteria per ml by plate count	Phage titre	Number of protozoa per ml	Number of bacteria per ml by plate count	Phage titre
24/6	498	12.000.000	750.000	10^{-2}	498	830.000	10^{-2}
25/6	0	10.000.000	680.000	10^{-1}	16.715	11.000	10^{-2}
26/6	0	8.400.000	221.000	10^{-1}	665	1.300	10^{-2}
27/6	0	8.000.000	64.000	10^{-1}	269	100	10^{-1}
28/6	0	36.000.000	18.000	10^{-1}	335	390	10^{-1}

Nothing points at an increase of the phage titre during the purification process of the water. The estimation of the titre in the KCN-free water was made after 24 hours, the contents of the bottle being clear. It might be conceived that in this experiment, like in those taken by SCHUURMAN with water from the Tjiliwoeng, the titre might have increased after 6 hours and decreased later on. In order to esteem this possibility another experiment was started.

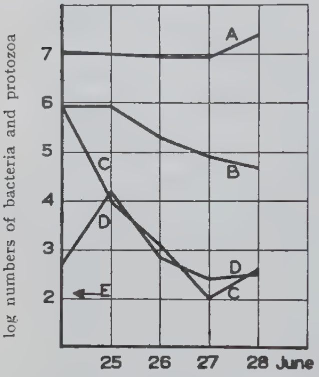

Fig. 1. Experiments with *Bacterium Shigae.*

A. Bacteria in water with KCN counted by PETROFF—HAUSSER apparatus.
B. Bacteria in water with KCN by plate count.
C. Bacteria in water without KCN by plate count.
D. Protozoa in water without KCN.
E. Protozoa in water with KCN.

6 bottles had been filled with 900 ml of Rhine water each. Every two bottles were supplied with 1, 2 and 5 ml respectively of a suspension in 17 ml of 0.9% NaCl of Shiga bacteria grown on agar slants during 18 hours. To every second bottle 40 mg KCN had been added. The number of protozoa amounted to 904. The bottles were kept at room temperature (22°C.) in diffuse daylight. After intervals of 6 and 24 hours the phage titre against Shiga bacteria was estimated; the number of bacteria was estimated after 24 hours (Table 4).

Table 4.

Treatment of liquids	Phage titre direct	Phage titre after 6 hours	Phage titre after 24 hours	Protozoa per ml after 24 hours
A with 1 ml Shiga suspension	10^{-2}	10^{-2}	10^{-1}	2.500
A′ ,, 1 ml ,, ,, + 40 mg KCN	10^{-2}	10^{-2}	10^{-1}	0
B ,, 2 ml ,, ,,	10^{-2}	10^{-2}	10^{-1}	4.391
B′ ,, 2 ml ,, ,, + 40 mg KCN	10^{-2}	10^{-2}	10^{-1}	0
C ,, 5 ml ,, ,,	10^{-2}	10^{-2}	10^{-1}	90.969
C′ ,, 5 ml ,, ,, + 40 mg KCN	10^{-2}	10^{-2}	10^{-1}	0

Again in the latter experiment nothing points to an increase of the phage. On the contrary the titre decreases with increasing purification.

The contents of the bottles without KCN were transparent after 24 hours when compared with those containing KCN, but a rich flora had developed in the former.

It remained to be investigated, whether KCN in the concentration which inhibits the development of the protozoa, would influence the activity of the phage. It has been recorded already that typhoid- as well as Shiga bacteria develop abundantly in broth containing 100—150 mg/l of KCN. After 24 hours at 37°C. the solution is equally turbid as broth without KCN.

A volume of sterile broth was provided with 50 mg/l KCN. To tubes with 5 ml of this solution typhoid- or Shiga bacteria grown 48 hours on agar slants were added. Two batches of these tubes were supplied additionally with a phage solution against these bacteria. Another batch was kept as control. The activity of the phage was moreover determined in pure broth. The results of this investigation indicate that KCN, even in concentrations 2 or 3 times higher than used in the experiment, does not act on the phage.

EXPERIMENTS WITH TYPHOID BACTERIA.

On July 8th an experiment was started with *B. typhi abdominalis*. Again there had been rainfall just before the starting of the experiment. Like in the former experiments two lots of 5 liters of Rhine water were put to the test. The number of protozoa in the water was 6146. Amongst these occurred many ciliates, *e.g.* vorticellidae. The algae occurred abundantly as well. 50, 25, 10, 2 × 5 and 3 × 1 ml were mixed with broth and inoculated with typhoid bacteria After 18 hours at 37°C. the cultures were filtered and the filtrates were examined for the presence of typhoid phage. The results were entirely negative. During the course of the experiment the examination for typhoid phage had been repeated after 24, 2 × 24 and 4 × 24 hours and at the close of the experiment. All results were negative.

One of the bottles was supplied with 50 mg KCN per l, the other was left without. Both bottles were provided with equal amounts of 18 hours cultures of *B. typhi abdominalis* (3 slants emulsified in 15 ml of 0.9% NaCl: each bottle received 7.5 ml). The results are recorded in table 5 and fig. 2.

On July 9th the liquid containing KCN is still turbid and remains so till the end of the experiment on July 15th. At the bottom of the bottle a green layer has settled which apparently consists of algae.

Table 5.

Date	With KCN				Without KCN		
	Number of protozoa per ml	Number of bacteria per ml counted by PETROFF-HAUSSER app.	Number of bacteria per ml by plate count	Phage titre	Number of protozoa per ml	Number of bacteria per ml by plate count	Phage titre
8/7	6.146	68.000.000	8.100.000	0	6.146	3.700.000	0
9/7	0	46.000.000	3.060.000	0	180.681	180.681	0
10/7	0	52.000.000	3.770.000	0	4.517	2.100	0
11/7	0	41.000.000	2.500.000	0	1.548	2.090	0
12/7	0	48.000.000	3.150.000	0	250	500	0
15/7	0	46.000.000	138.000	0	620	180	0

Remark: The liquids in both bottles were equally turbid on July 8th.

The liquid without KCN has cleared up already after 24 hours at room temperature (20°C.). After 24 hours the transparency is more marked and after 72 hours it is complete. After 4 × 24 hours many algae have developed. Along with the decrease in the number of protozoa, which had initially developed abundantly after inoculation with the typhoid bacteria, small crustaceae develop. These transparent animals may be easily studied in the dark field and it can be observed that their intestinal canal is filled with detritus, protozoa and algae. The impression is gathered that increase in the number of crustaceae follows increase of protozoa and algae, and that the former is the cause of the decrease of protozoa after the rapid development during the first 48 hours.

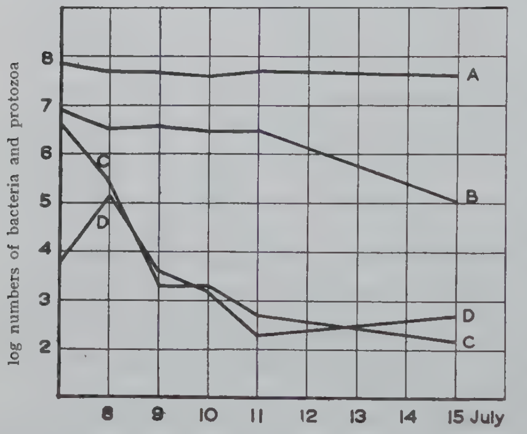

Fig. 2. Experiments with *Bacterium typhosum*.

A. Bacterja in water with KCN counted by PETROFF—HAUSSER apparatus.
B. Bacteria in water with KCN by plate count.
C. Bacteria in water without KCN by plate count.
D. Protozoa in water without KCN.

EXPERIMENTS WITH CHOLERA VIBRIOS.

Again Rhine water is investigated in two lots of 5 liters, one with and one without KCN. The phage titre on cholera vibrios is zero and remains so during the experiment. Each bottle has been inoculated with the suspension of one agar slant of vibrios. In both bottles the liquid is very turbid. For results see table 6 and fig. 3.

Table 6.

Date	Water with KCN				Water without KCN		
	Number of protozoa per ml	Number of bacteria per ml counted by **Petroff-Hausser** app.	Number of bacteria per ml by plate count	Phage titre	Number of protozoa per ml	Number of bacteria per ml by plate count	Phage titre
16/7	602	41.000.000	1.700.000	0	602	480.000	0
17/7	0	52.000.000	31.000	0	31.022	61.000	0
18/7	0	42.500.000	126.000	0	1.265	3.800	0
20/7	0	52.000.000	85.000	0	403	2.890	0
21/7	0	54.000.000	43.600	0	88	350	0

Already after one night the liquid without KCN had cleared up; after three days had elapsed it had grown completely transparent, whilst a layer of protophytes had settled on the bottom. The liquid containing KCN was as turbid at the close of the experiment as at the beginning.

The examination for cholera vibrios was made after the liquid had been kept for 72 hours; its results were negative. This was strange: in fact the number of cholera vibrios might be expected to increase in the bottle without KCN, but not so in the bottle containing KCN. This result, next to the fact, that the curve of bacterial numbers in the bottle with KCN, counted by the plate method was congruent with a like curve for the bottle without KCN, induced me to repeat the experiment, now extended with a dayly estimation of the cholera titre.

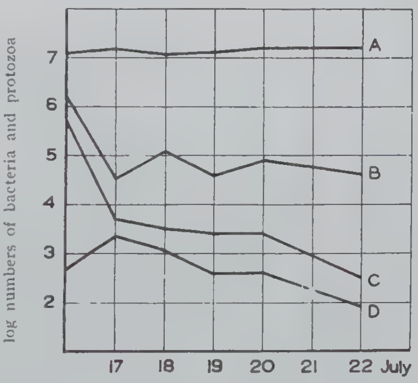

Fig. 3. Experiments with *V. cholerae.*

A. Bacteria in water with KCN counted by Petroff—Hausser apparatus.
B. Bacteria in water with KCN by plate count.
C. Bacteria in water without KCN by plate count.
D. Protozoa in water without KCN.

On July 24th 1940 once again the purifying action of the Rhine water on the cholera strain R.A.K. was investigated. Again two 4 liter lots of water were inoculated with 1.5 agar slants of cholera vibrios R.A.K., one lot being provided with KCN the other left without. The bottles were kept at room temperature (20°C.) in diffuse daylight. The results are recorded in table 7.

Table 7.

Date	With KCN			Without KCN		
	Number of protozoa per ml	Number of bacteria per ml by plate count	Phage titre	Number of protozoa per ml	Number of bacteria per ml by plate count	Phage titre
24/7	1.062	1.092.000	0	1.062	1.064.000	0
25/7	0	15.000	0	5.059	60.000	0
26/7	0	14.000	0	63.335	1.730	0
27/7	0	26.000	0	309	412	0

The cholera vibrios, which had been traced by direct plating on DIEUDONNÉ agar in both liquids at the beginning of the experiment, could at the close of the experiment no more be detected by means of enrichment cultures in peptone water and subsequent plating on DIEUDONNÉ agar even in 10 ml samples.

From this experiment as well as from the preceding one the impression is won, that the action of even low concentrations of KCN on cholera vibrios is more considerable than its action on Shiga- and typhoid bacteria.

If broth is supplied with 50 mg KCN per l, hardly any injurious action can be recorded. It might be conceived however, that injury would occur, when the vibrios were kept in water, which is a much less suitable medium.

In order to study this possibility dilutions of KCN in peptone water, amounting to 50, 40, 30 and 20 mg per l were made. Portions of 8 ml of these dilutions were transferred to small test tubes.These were supplied with a number of cholera vibrios, determined such as to allow of equal turbidity as the blancs. Moreover tubes with peptone water, provided with the tenfold dose of KCN as it was used in the experiment, were inoculated in the same way. The KCN solutions thus treated were kept at 37°C. and the growth of the vibrios was recorded (cf. table 8).

Table 8.

mg KCN per l in peptone water	Period of incubation			
	6 hours	24 hours	2 × 24 hours	3 × 24 hours
500	—	—	—	—
400	—	—	—	—
300	—	—	—	—
200	—	—	—	—
50	—	—	marked growth	
40	—	trace	profuse growth	
30	—	marked growth	profuse growth	
20	—	profuse growth	profuse growth	
0	profuse growth	profuse growth	very profuse growth	

Although neither an amount of 50 mg KCN per l inhibits the development of the vibrios, nor 20 mg per l materially influence it, growth appears to be impeded by 50 mg per l, whereas even 20 mg per l render the medium less suitable for growth than is the KCN free peptone water.

It has been stated before that KCN has no influence whatever either on Shiga bacteria or on typhoid bacteria. Neither can any unfavourable influence be recorded on their bacteriophages, when 50 mg KCN per l have been added to the broth. It seemed worth while, however, to repeat the experiments with Shiga bacteria whilst KCN was left out.

Protozoa are killed by the action of temperatures above 50°C., if applied during a sufficiently long period. Bacteriophages of Shiga-, typhoid- and cholera bacteria, however, require temperatures above 70°C. for their inactivation. Temperatures somewhat above 50°C. are therefore suited for the killing of the protozoa occurring in the water without impeding the activity of the phage eventually present.

A large volume of river water is kept at a temperature of 52°C. during 2 hours in a large electrically heated and well isolated water-bath; the water of the latter is kept in constant motion by means of a stirring apparatus, whilst its temperature remains constant within 0.25°C. All protozoa succumb during this treatment, as was learned by examination of the water.

5 liter lots of thus treated and untreated water (taken from the

„Galgenwater") are inoculated with the 18 hours culture layer of agar slants with Shiga bacteria and cholera vibrios, suspended in 5 ml of 0.9% NaCl. Both lots are kept in diffuse light at room temperature (15°—18°C.) and the self-purification is investigated as in the former experiments (see table 9 and 10 and figs. 4 and 5).

Table 9.

Purification of heated and non-heated river water from intentionally added Shiga bacteria.

Date	Heated water				Non-heated water		
	Number of protozoa per ml	Number of bacteria per ml counted by PETROFF-HAUSSER app.	Number of bacteria per ml by plate count	Phage titre	Number of protozoa per ml	Number of bacteria per ml by plate count	Phage titre
29/7	2.429	45.000.000	410.000	0.1 ml +	2.429	470.000	0.1 ml +
30/7	0	50.000.000	408.000		4.349	50.600	
31/7	0	55.000.000	3.980.000	1 ml —	1.136	3.160	1 ml —
1/8	0	52.000.000	4.010.000		4.703	296	
2/8	0	51.000.000	9.200.000		1.479	76	

Remark: On examination of 0.05 ml of the 1 ml concentrate from 250 ml of heated water there is not only complete absence of protozoa, but algae and higher plants and animals are lacking as well. Only decolorized scales of algae may be noted.

Table 10.

Purification of heated and non-heated river water from intentionally added cholera vibrios.

Date	Heated water				Non-heated water		
	Number of protozoa per ml	Number of bacteria per ml counted by PETROFF-HAUSSER app.	Number of bacteria per ml by plate count	Phage titre	Number of protozoa per ml	Number of bacteria per ml by plate count	Phage titre
29/7	2.420	42.000.000	60.000	0	2.420	240.000	0
30/7	0	59.000.000	1.600.000	0	3.692	39.200	0
31/7	0	60.000.000	1.560.000	0	5.491	2.800	0
1/8	0	60.000.000	1.860.000	0	2.994	324	0
2/7	0	60.000.000	1.640.000	0	2.325	240	0

On 29/7 cholera vibrios could be detected in 0.05 ml of either bottle and again on 31/7. On 1/8 the result was positive in 5 ml of the untreated water, negative in smaller samples; 1 ml of water which had been heated to 50°C. was positive, smaller volumes were negative. The bottles (2 with Shiga bacteria and 2 with cholera vibrios) were left in diffuse daylight at room temperature; the earliest possible date for a further examination was August 13th. On this date both lots of treated water were as turbid as at the beginning of the experiment whilst the non-treated water was entirely transparent with the usual layer of brown-green algae at the bottom of the bottles. 100, 50 and 25 ml of the water supplied with cholera vibrios gave negative results when investigated on the same date.

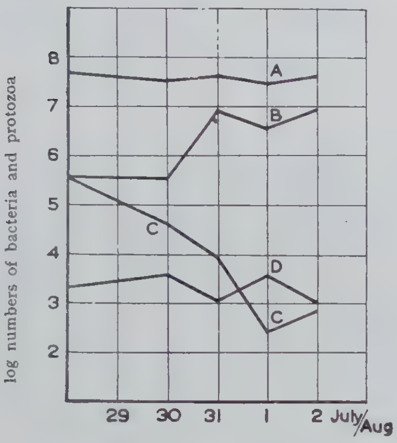

Fig. 4. Experiments with *Bacterium Shigae*.

A. Bacteria in heated water (52°C.) counted by Petroff—Hausser apparatus.
B. Bacteria in heated water (52°C.) by plate count.
C. Bacteria in non-heated water by plate count.
D. Protozoa in non-heated water.

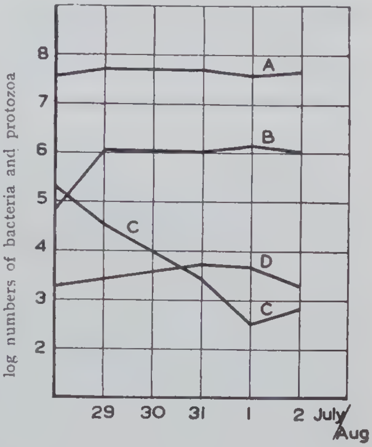

Fig. 5. Experiments with *V. cholerae*.

A. Bacteria in heated water (52°C.) counted by Petroff—Hausser apparatus.
B. Bacteria in heated water (52°C.) by plate count.
C. Bacteria in non-heated water by plate count.
D. Protozoa in non-heated water.

It could be learned from the examinations for cholera vibrios at regular intervals that these organisms vanish somewhat earlier from non-heated water than from water which has been freed from protozoa by heating. These results are at variance with those recorded in my earlier investigations at Batavia, where I noted cholera vibrios to remain alive during a long period in boiled water from the Tjiliwoeng. No further study of this discrepancy is possible, but I may emphasize the fact, that the water from the Tjiliwoeng has properties widely different from those of the Rhine („Galgenwater”) at Leiden. Whilst the latter is clear, colloidal clay makes the former strongly turbid.

Complete clarity cannot be attained in Tjiliwoeng water either by simple filtration or by means of bacteria candles.

On August 13th all four bottles were examined for protozoa as well as for bacteria. The results were:

Protozoa in river water provided with cholera vibrios		Protozoa in river water provided with Shiga bacteria	
non-heated	heated	non-heated	heated
877	0	981	0

Shiga phage in all lots was positive only in 2.5 ml.

The clear liquids rich in protozoa were added to the bottles with water turbid from bacteria and vibrios in such amounts, that the number of protozoa in the ,,vibrio water'' would attain 123 per ml and in the ,,Shiga water'' 110. The degree of turbidity was dayly recorded. On August 19th after an interval of 6 days the liquids were clear. The number of protozoa had increased from 123 to 5085 in case of the vibrios and from 110 to 1778 in case of the Shiga bacteria. In all liquids the phage titre against Shiga bacteria was estimated; results were negative in all samples below 5 ml. The number of bacteria in 1 ml was:

In the mixture of heated and non-heated water		In non-heated water	
cholera vibrios	Shiga bacteria	cholera vibrios	Shiga bacteria
70	361	21	20
The number of protozoa in 1 ml was:			
281	51	281	412

The coli count according to Eijkman was estimated in all liquids by examining 5 samples of 1 ml, 5 of 5 ml, 2 of 25 ml, one of 50 ml and one of 100 ml. The results were entirely negative safe in the bottle with the non-heated water provided with cholera vibrios, where the sample of 100 ml showed a trace of gas. At the opening of the experiment the coli titre had been positive in 0.001 ml.

Considering the results of these investigations my only possible conclusion is that under the artificial conditions which cannot be avoided in similar experiments, the bacteriophage does not take an important part in the purification of water. In the experiments with typhoid bacteria and cholera vibrios this influence has been completely excluded as no phages against these bacteria occurred in the water. Neither however did the Shiga phage, which was traced in 0.001 ml of natural water, multiply; on the contrary a strong decrease of the phage concentration has been recorded as the result of purification.

The experiments such as they have been carried out may be open to severe criticism in as far as water under natural conditions will never be so heavily contaminated as in my experiments. As phages develop most abundantly when small quantities of phage-susceptible bacteria have been added to the liquids, it may be conceived that the phage might increase when merely a small inoculum had been supplied to the river water.

So the following experiments were started: two lots of 4 liter of Rhine water were transferred to bottles of 6 liter capacity. One served as a blanc. To the other a loopful of an 18 hours culture of Shiga bacteria was added (table 11).

Table 11.

Date	River water without Shiga bacteria		River water with Shiga bacteria	
	Number of protozoa per ml	Phage titre against Shiga bacteria	Number of protozoa per ml	Phage titre against Shiga bacteria
21/9 11.30 a.m.	265	10^{-2} +	104	10^{-1} +
3.45 p.m.		10^{-1} +		10^{-1} —
22/9 11.30 a.m.	1.102	10^{-1} —	419	10^{-1} —
23/9	284		181	

Remark: The water did not turn turbid as a result of the inoculation and remained as transparent as in the blanc which was slightly opalescent.

After 48 hours at room temperature the contents of both bottles were completely clear and a layer of algae and detribitus had settled on the bottom. Table 11 shows that even inoculation with a small amount of bacteria does not induce after either 6 or 24 hours any increase in phage concentration of river water. Again in the latter experiment the number of protozoa has increased. This keeps true for the uninoculated blanc as well.

Several authors, *e.g.* KRUSE, who admit the significance of protozoa for the purification of heavily contaminated water, have claimed that their action is less preponderating as long as the contamination is moderate and corresponds better with natural conditions. The number of protozoa would not increase under such condition, at least no increase could be recorded.

The latter experiment proves however an increase to occur. The negative results of experiments with moderately contaminated water are probably to be ascribed to the experimental technique.

Summary.

As early as 1926 the present author has claimed that in the self-purification of water (under experimental conditions realized in the laboratory) bacteriophages take no part, at least not an easily discernable one. The investigations of Schuurman on the significance of typhoid phages in the self-purification of the water in the Tjiliwoeng river induced the author to attack the problem once more from the experimental side.

The results of these recent investigations allow of no other conclusion than that under circumstances and conditions as have been fully described, bacteriophages do not act on the self-purification of water.

It might be conceived, that the high temperature of the Tjiliwoeng river and its high content of colloidal clay allow the phage to play a certain part, although the results of the author's experiments make this hardly probable. At all events such an influence is neither proved nor made probable by the results of Schuurman's experiments with the untreated Tjiliwoeng water, because of his failing to exclude the action of the protozoa.

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SOME EXPERIMENTS WITH BLOWN GLASSES

by

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Since the 300th anniversary of the birth of ANTONIE VAN LEEUWENHOEK in the year 1632 there is a growing interest in the works of this remarkably keen observer. A valuable contribution to our knowledge of VAN LEEUWENHOEK was given by DOBELL (7) in his book: Antony van Leeuwenhoek and his ,,Little Animals". DOBELL drew the attention to the question how it was possible that VAN LEEUWENHOEK was able to describe protozoa and bacteria so well, as he had but very primitive microscopes to observe them with.

Some time ago I made some incidental observations with a small blown glass sphere and the results I got with this lens induced me to carry out some further investigations. It is generally believed that simple lenses have not much value: they would give but poor results, as lenses with high magnification have much spherical and chromatic aberration because of their strongly curved surfaces. DOBELL (7) for instance writes: ,,Yet LEEUWENHOEK not only knew how to make lenses of adequate magnifying power and aperture and resolution and sufficiently free from spherical and chromatic aberration, but he also understood how to obtain the necessary visibility". So DOBELL suggests that VAN LEEUWENHOEK did not grind spherical surfaces, but some other curved surface that had no spherical aberration.

It was DESCARTES (6) who tried to solve this problem. Theoretically he succeeded, but it is not very probable that in practice he had any success. Still even nowadays it is an extremely difficult job to make other than spherical surfaces. As for the chromatic aberration, this cannot be eliminated when only one sort of glass is used.

Meanwhile it remains very remarkable that VAN LEEUWENHOEK had such skill in grinding that he was able to make such extremely small lenses. No doubt his skill surpassed by far that of his contemporaries. Instead of ground glasses often small glass spheres, so-called blown glasses, were used. van Leeuwenhoek however displayed a great contempt for these blown glasses as may be learned from a conversation he had with von Uffenbach (cf. (7), pg. 68). Further on we will see that there is not much reason for such a contempt.

The most simple method to make such a blown glass certainly is the one used by Hooke (8), which was also put into practice by Hartsoeker, Musschenbroek and other investigators. First a very thin glass thread is drawn, the end of which is introduced into the blow-flame for a few moments so as to obtain by melting a small glass sphere. This sphere is broken off and it is of no consequence that it carries a small piece of the original thread. It is then mounted between two brass plates, each with a small hole in it, in such a way, that the shaft points aside. Both van Cittert (1) and me used this method with good results. The images obtained with such lenses really are astonishingly good. They are remarkably free from spherical and chromatic aberration and even with a magnification of 500 times they still give the impression of being practically achromatic. Cohen (5) as well states that unground lenses are very useful.

Harting (8) also made small glass spheres, but his method was somewhat more complicated. His lenses had a magnification of 80 to 2000 times. Harting was of the opinion that the clearness of the images he obtained with his spheres could be only explained by supposing that the spheres had not a purely spherical surface, but either a hyperboloidical or an ellipsoidical one. This of course cannot be the right explanation. According to van Cittert (3, 4) theoretical considerations would make it clear, that in the case of strongly curved, nearly spherical lenses the deterioration of the quality of the image will be due more to the deviation from flatness than to the spherical and chromatic aberration, which will affect the image only very slightly.

The results I got with my first small blown glass induced me to try which ultimate results might be obtained with such glasses. Therefore I made a series of small spheres with different diameters. Nearly all spheres appeared to be good; a few ones which did not serve were spoiled by small airbubbles. This can be avoided by using not too hot a flame.

In order to take photographs, I cemented with resin the small copper plates to the upper end of an old-fashioned objective. In this way it is possible to fit the small lens to an objective slide and to adjust it to the center of the illuminating condensor. The ocular part of the microscope was replaced by the metallic bellow of a Zeiss „Miflex". As illuminating unit was used a handfeed arclamp, using a 5 amp. alternate current. The upper part of the microscope condensor was removed.

The following range of lenses was made:

Lens nr.	diameter	magnification at 20 cm	magnification at 25 cm
1	2.0 mm	133	166
2	1.75 mm	150	187
3	1.50 mm	175	218
4	1.20 mm	210	250
5	1.00 mm	220	275
6	0.85 mm	300	375
7	0.85 mm	300	375
8	0.75 mm	350	437
9	0.60 mm	460	575

I did not try lenses smaller than nr 9, because such ones would not be useful. The focus distance of a lens is 1.5 R, which means, that the distance of the focus and the surface of the lens is 0.5 R. For lens nr 9 this is 0.15 mm, which is about the average thickness of a coverslip.

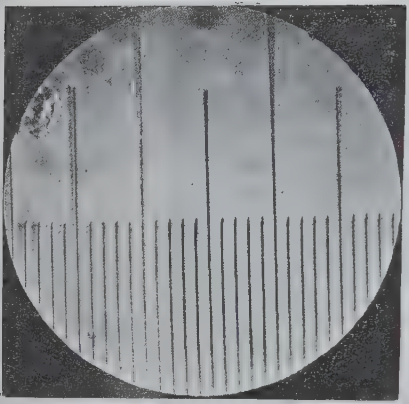
It appears clearly from the reproduced photographs, that the single spherical lens attains at a fairly high achievement.

Plate I. Object micrometer taken with lens nr 3, 5, 6, 7, 8 and 9.

Plate II. *Triceratium venosum* taken with lens nr 1, 2, 4, 7, 8 and 9.

Plate III. Taken with lens 1, magnification 133 ×.
Fig. 1. sting of a bee.
Fig. 2. tongue of a bee.
Fig. 3. wing of a bee.
Fig. 4. *Radiolaria.*
Fig. 5. scales upon the wing of a butterfly (*Limantria dispar*).
Fig. 6. legs of a flea.

I



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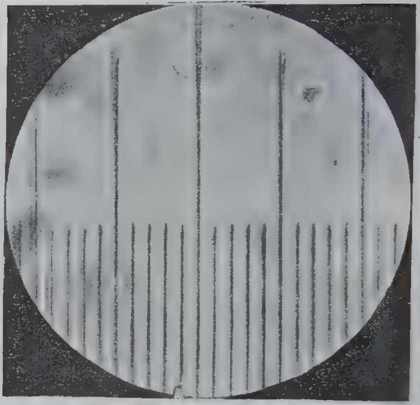

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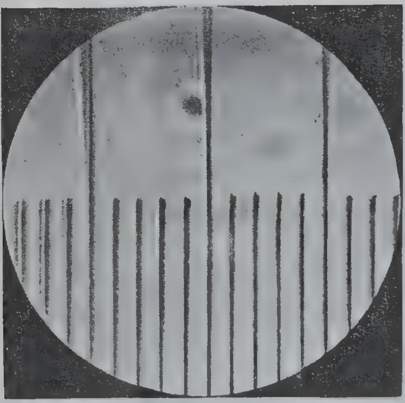

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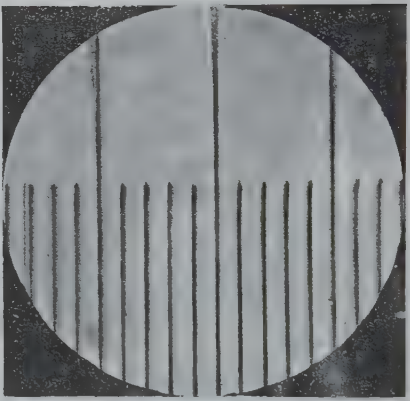

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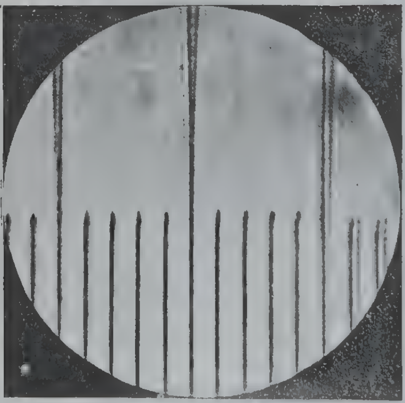

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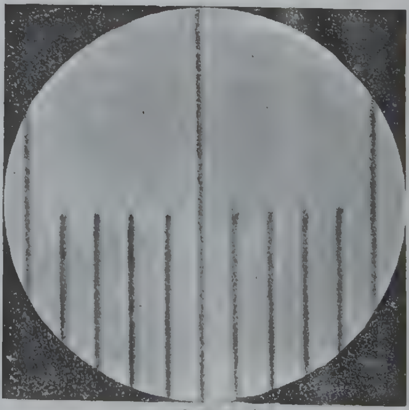

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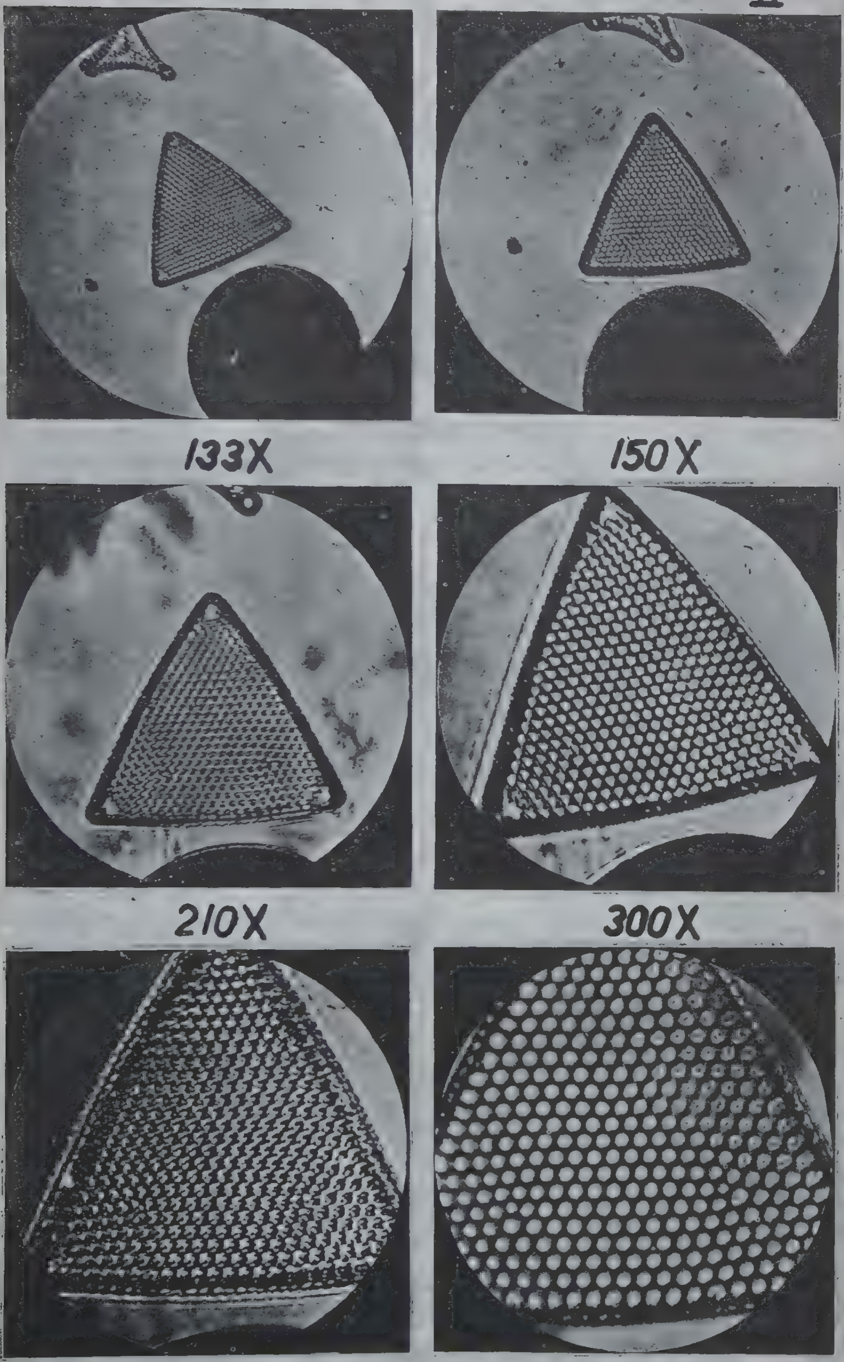

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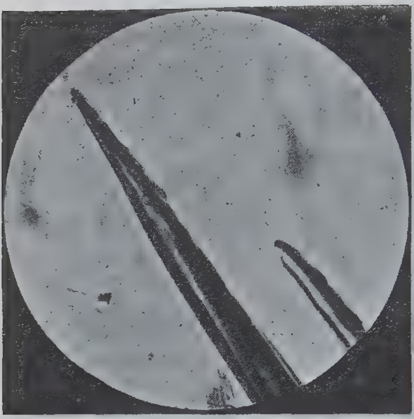
210X

300X

350X

460X

III



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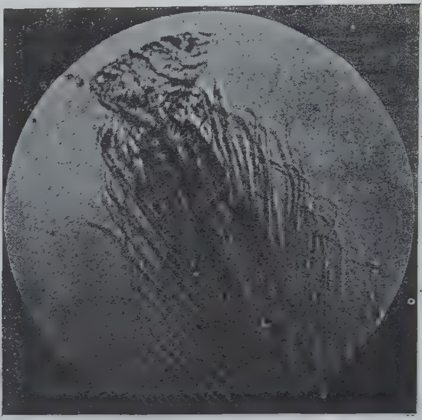

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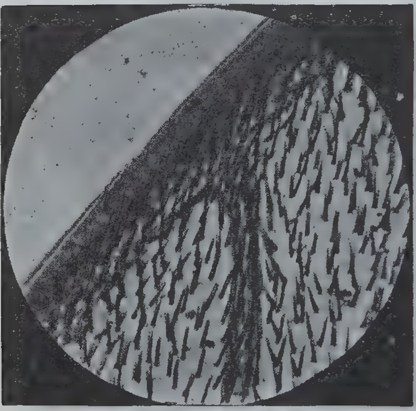

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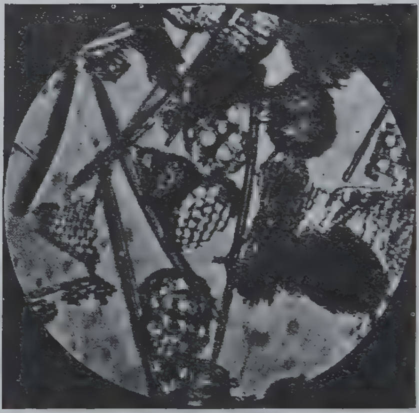

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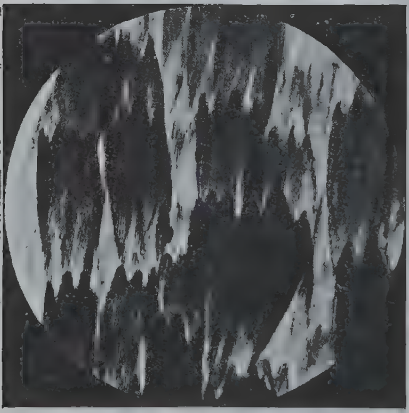

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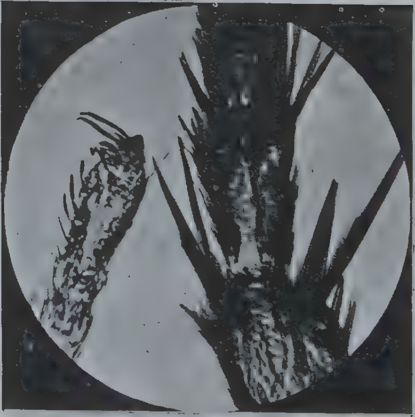

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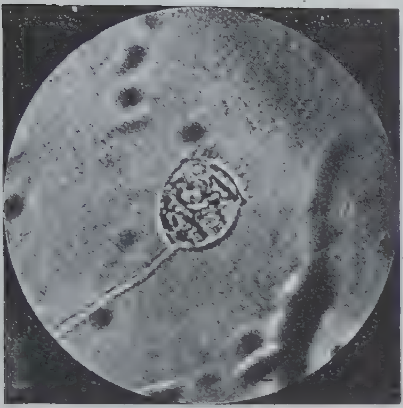

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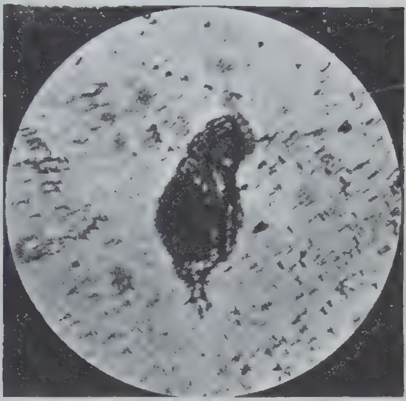

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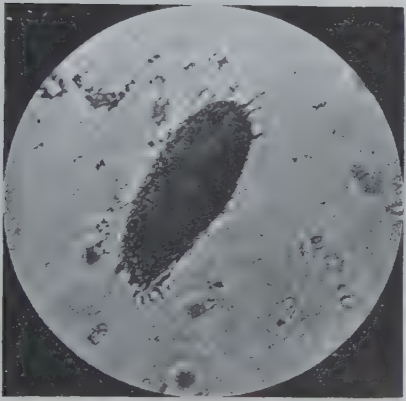

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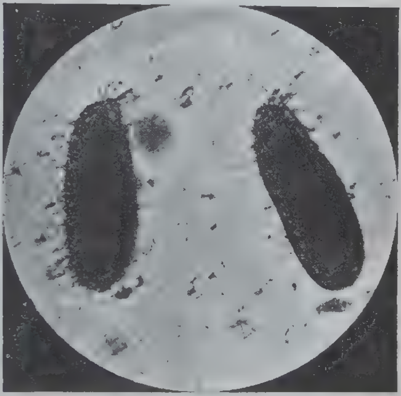

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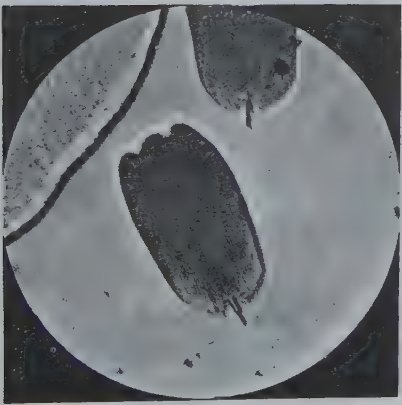

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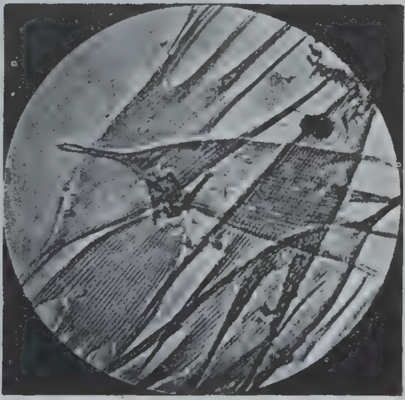

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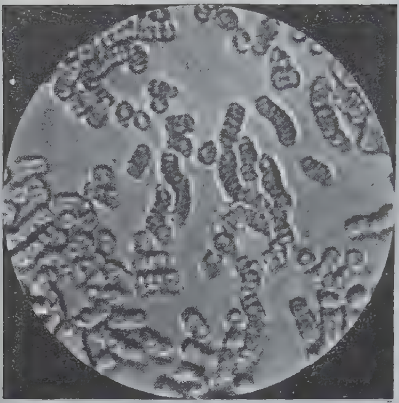

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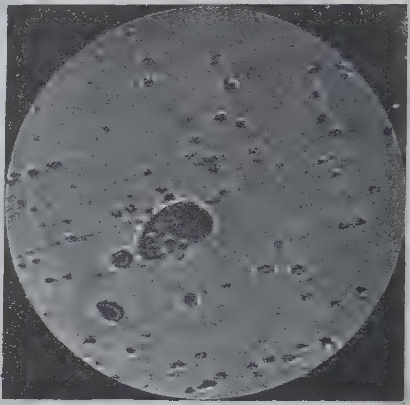

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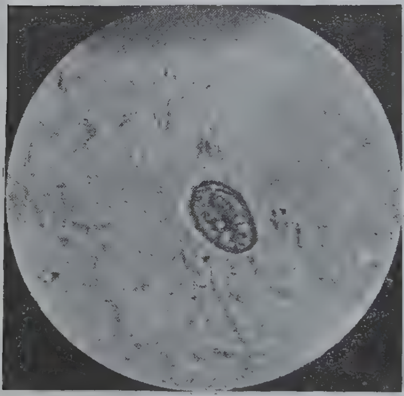

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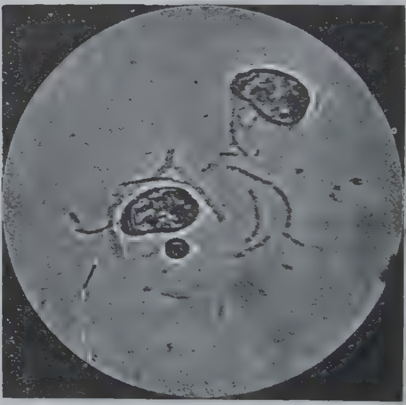

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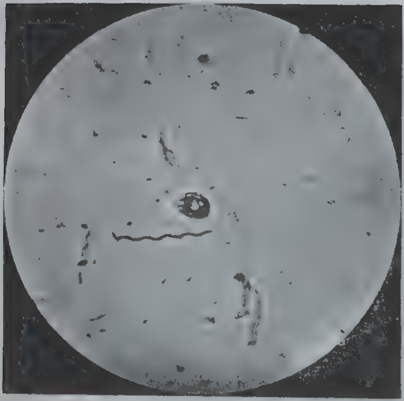

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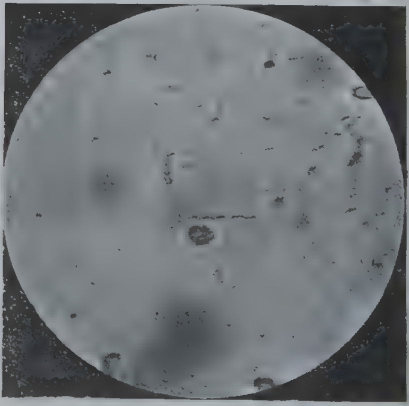

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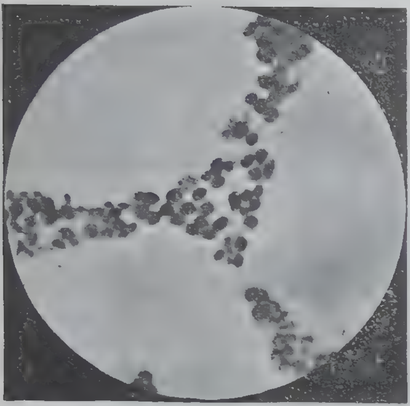

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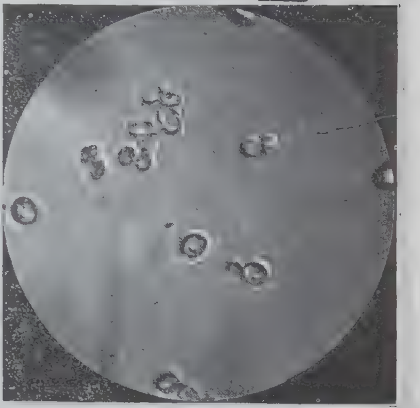

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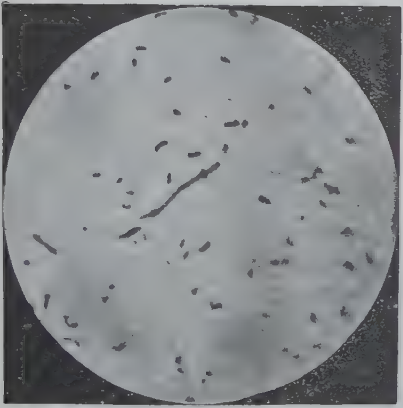

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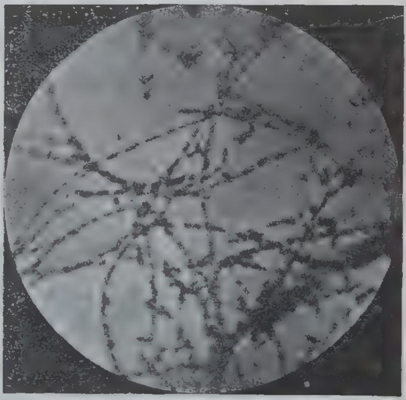

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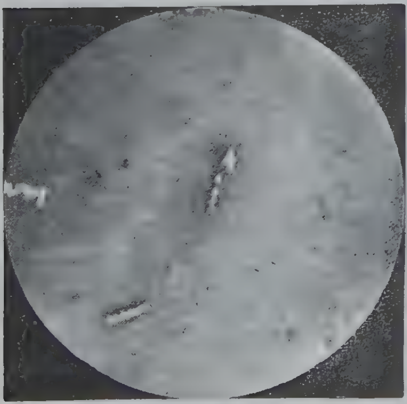

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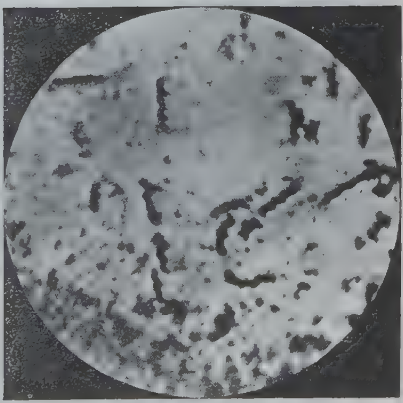


6

Plate IV. Taken with lens 4, magnification 210×.
Fig. 1. *Vorticella alba.*
Fig. 2. *Rotifere.*
Fig. 3. *Paramecium* spec.
Fig. 4. *Paramecium* spec.
Fig. 5. Scales of the wing of a butterfly (*Aglais urticae*).
Fig. 6. Scales of the wing of a silk-worm moth (*Bombyx mori*).

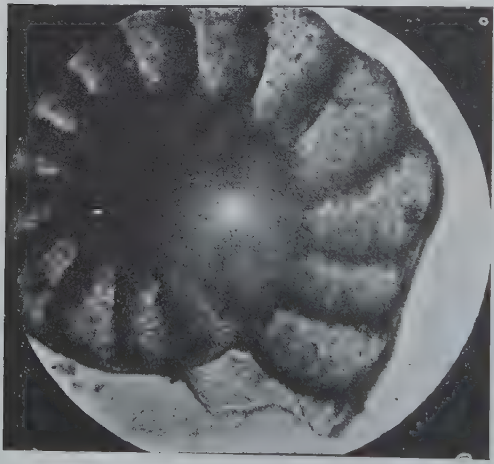
Plate V. Taken with lens 6, magnification 300×.
Fig. 1. Red blood corpuscles (man).
Fig. 2. *Colpoda* spec., flagella faintly visible.
Fig. 3. *Colpidium* spec., flagella faintly visible.
Fig. 4. *Colpidium* and bacteria.
Fig. 5. *Chlamydomonas* spec. with two flagella and a *Spirillum.*
Fig. 6. Same as 5, but taken with lens 4, magnification 210×.

Plate VI. Taken with lens 9, magnification 460×.
Fig. 1. *Saccharomyces cerevisiae,* sporestaining, cell with 4 spores.
Fig. 2. *Saccharomyces cerevisiae,* unstained preparation.
Fig. 3. *Streptococcus lactis,* Gram staining.
Fig. 4. *Sphaerotilus natans,* carbol fuchsin staining.
Fig. 5. *Bacillus mycoides,* sporestaining Dorner, 4 spores.
Fig. 6. *Spirillum* spec., Gray staining.

Considering this some questions rise. Primarily, did VAN LEEUWENHOEK really grind his small lenses, secondly, is there any reason to grind such lenses and finally, if VAN LEEUWENHOEK did grind his lenses, what induced him to do so.

Fortunately some of his microscopes have been preserved. Comparing the diameter of the lenses of these microscopes with their radius of curvature, we find that this diameter is more than twice the radius (4, 10). Therefore these lenses certainly have been ground.

The lens of the microscope at Utrecht has a magnification of 270×. Its diameter is 1.1 mm and its radius of curvature 0.75 mm.



Foraminifere 100×.

To answer the second question we must consider Fig. 1, which is partly taken from VAN CITTERT (3, 4). In this figure the resolving power is plotted against the magnification. It is very interesting to note that up till 1830 the compound microscope was surpassed by the single microscope, the latter having a better resolution at the same magnification. At about 1830 the single microscope lost its leading position and nowadays it is used only as magnifying glass.

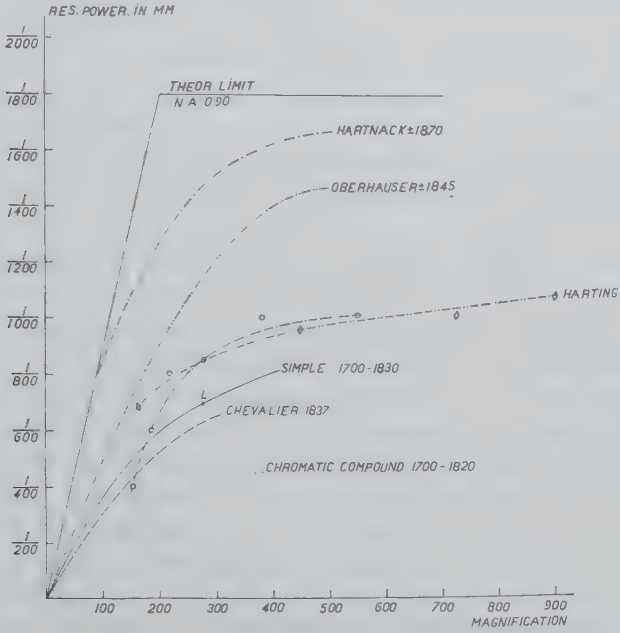


Fig. 1.

HARTING (8) determined the ratio for his glasses with the aid of a NOBERT plate and so it was possible to draw the curve (HARTING) in the graph. I did the same thing for my glass spheres with a GRAYSON's ruling (4). The result was the curve marked O. In the museum of the University of Utrecht there still is a specimen of a GRAYSON's ruling. The close correspondence between the spheres of HARTING and mine is striking.

From this graph it appears, that the so-called blown glasses, magnifying more than 175 times, are certainly not inferior to the ground glasses of the same magnification. If the lens of VAN LEEUWENHOEK marked L in Fig. 1 had not been so badly scratched, its position in the graph might have been better.

On the other hand it can be observed, that the ground lenses with low magnification are certainly better than the blown ones. HARTING is of the same opinion. Perhaps this is the reason why VAN LEEUWENHOEK ground his lenses. When VAN LEEUWENHOEK started his observations, he probably did not immediately want lenses with high magnification, as the existence of micro-organisms was still unknown to him. Evidently he initially has aimed at moderately magnifying lenses, which he only could obtain by grinding, and when he finally had mastered this difficult art it is only natural, that he would stick to it.

I thought it interesting to repeat some of the observations made by VAN LEEUWENHOEK, especially in connection with a question raised by DOBELL, who remarks, that for observing bacteria and flagella and cilia of protozoa it is necessary, that there is some contrast between the object and its background. Although VAN LEEUWENHOEK did not know the nowadays much used stops, irisdiaphragms and different staining methods, yet he was able to get this contrast in some way or another. DOBELL is of the opinion that VAN LEEUWENHOEK could not have made his extraordinary observations of bacteria and protozoa by means of the generally known primitive microscopes, if he had used them merely in the ordinary way. Now we find in one of VAN LEEUWENHOEK's letters dated 22.1.1675 (7, 9) the following passage: ,,presently I can show to myself the globules of blood etc. as sharp and clear as one can distinguish with ones eyes without any help of glasses sandgrains, that one might bestrew upon a piece of black taffety silk". From this passage DOBELL draws the conclusion, that VAN LEEUWENHOEK must have known dark-ground illumination, else it would be difficult to understand, how he came to such a simile.

Meanwhile this simile can be conceived as just a phraseology by which VAN LEEUWENHOEK has meant to convey the impression of the extraordinary clearness with which he saw the red globules in the blood. How this may be, DOBELL's clever suggestion has drawn attention and has been seriously considered by some investigators.

I learned from the experience I gathered with blown glasses, that it is not at all difficult to attain the necessary contrast and in my opinion VAN LEEUWENHOEK's chief difficulty must have lain in the fact that he had to observe all his organisms in capillary glass tubes. Especially the larger organisms are difficult to observe. Not only there occurs a rather serious distortion, but neither is it easy to get them into view. It is difficult to observe for instance the shapes of red blood corpuscles and of yeast cells in capillary tubes. The reproduced photo's clearly show that with direct illumination there is enough contrast to see bacteria, flagella and cilia with a simple lens. The magnification of plate IV is less than 275 times, the actual magnification of the van Leeuwenhoek microscope at Utrecht, whereas that of plate V is somewhat higher, namely 300 times. The photo's do not show everything as clearly as direct observation might. To arrive at an image of equal quality in capillary tubes proved to be much more difficult as can be seen on the photo's of protozoa made by van Cittert (1, 2).

It is much easier to make observations with a simple lens than one would expect at first view. To do so the slide is taken in the left hand and with the right hand the slide and the microscope are pressed together between thumb and fore-finger. After some exercise it is a fairly simple matter to focus and it is even possible to follow motile organisms.

In this way were observed:

With lens 1: Cilia of *Vorticella alba* (50 µ) and the currents caused by them. Cilia of a *Paramecium* species (150 µ). Grayson's ruling group 2.

With lens 2: *Leptothrix buccalis*. Grayson's ruling group 3.

With lens 3: Pulsating vacuoles of a *Colpoda* species (50 µ). The undulating membrane of the cytostome of a *Colpidium* species (50 µ). *Trichomonas*, an organism a little larger than *Giardia*, which van Leeuwenhoek, according to Dobell, would have observed in his faeces. Grayson's ruling group 4.

With lens 4: Movement of amoeba (*Vahlenkamfia* 10 µ). The typical shape of a *sarcodina* (10—12 µ). Bacteria in pepper water. Grayson's ruling group 4.

With lens 6: Structural difference between the ectoplasm of the pseudopodia and the endoplasm of the *Vahlenkamfia*. *Saccharomyces cerevisiae* and red blood corpuscles. Endospores in bacteria from hay-infusion. Lines on *Pleurosigma fermosum* (distance between the lines about 1 µ).

With nrs 8 and 9 of course everything can be seen much better. With nr 9 one can see very distinctly, that the ectoplasm of the pseudopodia of *Vahlenkamfia* is quite clear, whereas the endoplasm contains many very small granulae.

My experiments led me to the conclusion, that even if VAN LEEUWENHOEK had known the dark-ground illumination, it would have been of very little use to him as the one-sided illumination causes distortion. VAN CITTERT (1, 2) has arrived at the same conclusion. When slides and coverslips are used, the dark-ground effect obtained by oblique illumination is much better than in capillary tubes. The lower the magnification of the lens, the less oblique the illumination has to be to induce a dark-ground effect and the lesser will be the distortion. This explains why VAN SETERS (11), who used a lens, magnifying merely 40 times, considered the results, he got with dark-ground illumination fairly satisfactory.

With dark-ground illumination I could see the cilia of *Vorticella* and *Paramecium*, but I could observe them at least as well with direct illumination.

My second conclusion is, that VAN LEEUWENHOEK must not necessarily have had better microscopes than the one in charge of the museum of the University of Utrecht to day. I am even inclined to suppose, that it is not impossible, that by chance it might be the best microscope, VAN LEEUWENHOEK ever made, which has been saved. VAN CITTERT is of the same opinion [1]).

It has to be kept in mind, that the instrument is not the only factor determining the possible achievement, but the personal ability of the person using it is of no less importance. VAN LEEUWENHOEK was not only a very keen observer, but he also was very skilled in preparing his objects (7) and of course this too plays an important part.

It may be further observed, that VAN LEEUWENHOEK saw his objects more enlarged than other people looking through the same microscope. VAN SETERS (11), who drew the attention to this fact, cites as example, that VAN LEEUWENHOEK could see the longitudinal lines on the scales of the wing of a silk moth, which his draughtsman evidently could not perceive. This was the reason why I took some photo's on the scales on the wing of a butterfly. VAN LEEUWENHOEK apparently had not mounted the scales on a microscope with a strong lens, for at a magnification of 113 times we can already distinguish lines which occur much more closely. With a 210 × enlargement we can even perceive the transverse lines in the scales of another butterfly.

The latter considerations may make it obvious, that were there actually some inconceivable fact, it would be that van Leeuwenhoek made his remarkable observations in capillary glass tubes. If it were possible to show, that he has known the coverslip and actually used it, things would grow much clearer.

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[1]) Private communication.

In his description of microscopes it is with very good reason that van Cittert (3, 4) emphasises the significance of Amici, who was the first to draw the attention to the part, played by the coverslip. In this connection the following sentence of the already mentioned letter of van Leeuwenhoek of 22.1.1675 is of importance. To defend his method for observing the globules in blood he writes in this letter: ,,.... and such glass tubes now are rarely or not used. At present I have quite another method of observation for some time which is as good as (with improvements) can be invented and if I wanted this to reveal to the world, I do not doubt that you and all the Gentlemen Amateurs would esteem my instruments and method of observation" (cf. (9)). So here van Leeuwenhoek declares emphatically the fact, that he no longer uses capillary glass tubes. Whether he really used coverslips is not certain of course. It is quite possible, however, as he now and then fixed his preparations upon thin mica plates or very thin blown glass (cf. (7), pg. 317). Moreover he continues: ,,.... and it is curious to see how the globules in the blood, when they lie scattered, move towards each other". This suggests, that van Leeuwenhoek saw the bloodglobules lying in a flat plane.

In this connection another quotation from a letter of van Leeuwenhoek, dated 9.10.1678, too is of some importance. ,,My method for seeing the very smallest animalcules and the little eels I do not impart to others, nor yet that for seeing many animalcules at once, but I keep that for myself alone" (cf. (7), pg. 144). From this one would conclude, that he had two different methods of observing. If that were the case, the latter part of the above sentence might suggest, that he used coverslips, for this makes it possible to see many organisms at the same time. van Cittert (2) points out, that in the meniscus in capillary tubes the micro-organisms can be observed rather easily and he supposes that perhaps the quoted sentence may refer to this. It is however not possible to arrive at a definite conclusion in this matter.

It is worth mentioning that van Leeuwenhoek apparently was not quite satisfied with his new method, as he makes the restriction ,,with improvement". This may as well refer to the fact, that his microscopes were not well adapted to make use of a slide with a coverslip, as to the difficulty, that his preparations did not keep very well, at least not as well as in capillary tubes. This is very important, because van Leeuwenhoek had the habit to observe repeatedly the same preparation before he finally wrote down his observations. This method of working may partly have been due to the fact, that in the long run it is rather trying to use a simple lens.

How this may be, the actual methods of van Leeuwenhoek, which he kept so carefully to himself, in all possibility never will be known to us. If however one has experimented with simple lenses for some time, one arrives at the conclusion, that it is quite possible, that van Leeuwenhoek made his observations with the aid of his well known type of microscopes used in the usual way.

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(From the Laboratory of Hygiene of the University, Utrecht and from the Research Laboratories of N.V. Orgachemia, Oss).

ON THE ACTION OF SULFANILAMIDE

III. THE DIFFERENCE BETWEEN SULFANILAMIDE AND SULFAPYRIDINE [1]

by

H. W. JULIUS AND A. SALOMON [2]

(Received May 24, 1941).

It is beyond doubt, that for the treatment of a number of infectious diseases sulfapyridine (para-amidobenzene-sulfonamidopyridine, Dagénan, M & B 693) is a more potent drug than sulfanilamide. Whether it is justifiable to substitute sulfapyridine for sulfanilamide in all cases, is a matter open to discussion. But though we may be sure, that results can be obtained with sulfapyridine (and analogous preparations as sulfathiazol) that can not be reached with sulfanilamide, we are still groping in the dark as to the cause of this difference in effect.

It is the object of this article to contribute towards our knowledge of this remarkable group of drugs. Maybe, it will also show some new ways to further improvement. We will, however, limit ourselves to the question: What is the difference between sulfanilamide and sulfapyridine?

We can make two alternative suggestions:

1. Sulfapyridine is a substance, chemically related to — but as a drug essentially different from — sulfanilamide.
   More or less by chance, it has been an ,,other discovery".
2. In its action sulfapyridine is essentially the same as sulfanilamide, but the introduction of the pyridine radical has:
   - *a*) enhanced the action of sulfanilamide,
   - *b*) given a supplementary effect to the action of sulfanilamide.

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[1] Second communication: H W. JULIUS and K. C. WINKLER, Antonie van Leeuwenhoek **7**, 25, 1941.

[2] We are greatly indebted to the editors of ,,Antonie van Leeuwenhoek" for their kindness of accepting this paper for publication. It had been sent April 1940 to the ,,Lancet". We are not aware however whether it actually has appeared. It is a great satisfaction to the authors to be able to publish it nevertheless.

As to the latter of these suppositions (2*b*), the question arises what might be the nature of this supplementary action?

To decide between the main alternatives (1 and 2), it will be necessary to point out a feature characteristic of sulfanilamide, which might indicate whether a substance is either different from, or comparable to sulfanilamide in regard to its effect on bacteria. It is the general opinion that the action of sulfanilamide is only a bacteriostatic and not a bactericidal one. It has been pointed out by the late Professor WOLFF and one of us (1), that this holds true only in so far as sulfanilamide is not in any way comparable to other desinfectants, such as phenol, or even trypaflavin. Simple bacteriostasis however, does not exactly describe the action of the substance. For whenever sulfanilamide acts in vitro on bacteria (streptococci), there is an incubation period. This is not a mere question of time, but the length of this period depends on the number of bacterial cell divisions that have occurred in the presence of sulfanilamide.

The substance is, as far as can be concluded from serial countings, inactive if the divisions are not numerous enough, and if they are not frequent enough. The curves showing the growths with and without sulfanilamide run parallel during the first 5 or 8 divisions (fig. 1, curves *a* and *b*), *i.e.* during the period that the logarithm of the numbers is increased by 1.5 to 3. Then the growth curve of the culture containing 0.015% sulfanilamide (curve *b*) bends towards the horizontal, whereas the control curve (curve *a*) continues to rise. A desinfectant such as phenol, and even the chemotherapeutic trypaflavin, on the other hand, acts immediately (fig. 1, curves *c*, *d* and *e*). In the case of these two antiseptics it depends on the concentration, whether the slope of the curve

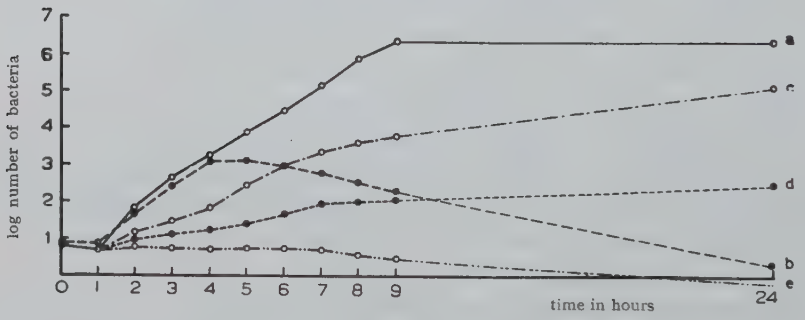

Fig. 1.

Curve *a*: normal growth curve.
Curve *b*: growth curve in a culture containing 0.015% sulfanilamide.
Curve *c*, *d*, *e*: curves in successive concentrations of phenol or trypaflavine (schematically).

will merely diminish (curve *c*), whether the curve will continue on the same level (curve *d*) or will even bend downward (curve *e*).

That these phenomena are essential is demonstrated by the fact, that an increase of sulfanilamide up to twenty times and more the minimum active concentration does not change the shape of the growth curve, whereas twice or thrice the minimum active concentration of an antiseptic (trypaflavin) promptly changes the direction of the curve (fig. 1, *c*, *d*, *e*). The term of true bacteriostasis should be reserved to the effect of the actual concentration of an antiseptic that changes the curve's direction almost to the horizontal, immediately after it has been added to the culture. We concluded from these facts that sulfanilamide must be a special „poison" to bacteria acting on the mechanism of their division. Characteristic of its action is a growth curve of the shape reproduced in fig. 1, curve *b*.

When sulfapyridine to a concentration of 0.015% has been added to a culture of streptococci, there is no difference between its growth curve and that of a culture to which sulfanilamide to the same concentration has been added. We made a good many tests of this kind with streptococci and pneumococci. Details of these experiments will be published later. They are only mentioned here in order to furnish proof that nr. 2 (cf. page 77) should be considered the most likely alternative.

This is, after all, quite in accordance with expectations founded on clinical observations, and experiments in vivo as well as in vitro, known from literature on the subject. These experiments lead to the conclusion that both substances belong to the same type of chemotherapeutics.

Experimental evidence (also to be published) enabled us to decide between the secondary alternatives as well. By varying the concentrations added to the cultures of pneumococci and streptococci, we have ascertained that differences in activity — if existing at all — are so small, that alternative 2*a* may be abandoned.

Our conclusion would logically read as follows:

that sulfapyridine acts like sulfanilamide, and that, probably accidentally, the introduction of the pyridine radical in the molecule causes some supplementary activity. In view of the fact that the experiments made by ourselves, as well as the literature we studied on the subject do — so far — not give the least indication as to sulfapyridine in vitro having another effect on bacteria than sulfanilamide, we are inclined to assume that this supplementary faculty acts either on the living, infected host, or — if on the bacteria — then only through the medium of the host tissues. Sulfapyridine is a substituted sulfanilamide, but now that we have assumed that it has an additional and perhaps unrelated effect on the living body, we may consider the substance as a pyridine derivative as well. It is therefore reasonable to try and ascertain whether sulfanilamide may be supplemented by some other substance so that its effect might come up to the level of sulfapyridine.

For this purpose an experimental infection was needed, that could be cured by sulfapyridine, but not by sulfanilamide. To a certain degree the pneumococcus meets this requirement. It should be mentioned however, that when comparing the effect of sulfanilamide with that of sulfapyridine on mice infected with different strains of freshly isolated pneumococci, there will, in several instances, be no indication of the desired difference. Sulfanilamide alone can cure animals infected by many of the strains; in other cases only a minor difference in effectiveness between the two substances could be ascertained. But by trying different strains of pneumococci, we succeeded in finding some strains that met our requirements. These will be dealt with in the following experiments. We would only point out that the claims for the superiority of sulfapyridine may not prove to be so far-reaching as is generally stated or accepted.

Sulfapyridine being considered a pyridine derivative, the question now arises, what is the action of pyridine? 30 mice were infected, intraperitoneally, with 10^{-5} ml of a 24 hours' old culture of pneumococci in 5% blood broth medium. 6 served as controls, 6 were treated with sulfanilamide, 6 with sulfapyridine, 6 with pyridine, 6 with a mixture of sulfanilamide and pyridine. During the experiment the drugs were administered in half doses twice daily (Table 1).

Table 1.

preparation	daily dose in mg	number of animals	dead on					survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day		
controls . .	—	6	6	—	—	—	—	—	1.0
sulfanilamide	10	6	—	—	6	—	—	—	3.0
pyridine . .	10	6	5	1	—	—	—	—	1.1
sulfanilamide + pyridine . .	10 + 10	6	—	1	1	2	2	—	3.8
sulfapyridine	10	6	—	—	—	2	3	1	4.8

Excepting the case of one animal, the dose of sulfapyridine in this experiment proved too weak to cure the infection. The addition of pyridine, however, clearly improved the action of the sulfanilamide.

This may be less apparent when judging by the figure indicating the average time of survival, than from the general condition of the animals, *e.g.* on the second day, and the prolongation of the life of some of them.

This very simple experiment was repeated several times, with the same result. In order to ascertain, whether the way in which pyridine was administered was of any influence, we gave in a second experiment the sulfanilamide per os, the pyridine subcutaneously (Table 2).

So the addition of a per se inactive substance will increase the therapeutic effect of sulfanilamide on infections that are sulfanilamide-resistant but can be cured by sulfapyridine. The latter substance is, however, far from being equalled. Experiments in vitro (still to be published) again pointed in the direction that pyridine — either when administered singly, or combined with sulfanilamide — does not act on the bacteria, at least not in concentrations as may be expected in vivo.

The possibility of sulfapyridine being synthesised from the two substances by the action of animal cells — though rather improbable — had to be considered. We therefore tried α-amido-pyridine singly and in combination with sulfanilamide, but the toxicity of the substance was too high. The admissible dose (2 mg twice daily) proved inactive.

Neither did β-pyridine-carboxylic acid (nicotinic acid) alter the action of sulfanilamide (2). On the other hand, the effect of α-methyl-pyridine (picoline) proved to be of the same order as that of pyridine, so a synthesis could be excluded. This led to the supposition that it might be the α-pyridine compound that enhances the action of sulfanilamide. So, step by step, we followed the synthesis of pyridine to α-pyridine-p-sulfonamide-benzene-amid (sulfapyridine).

All experiments were made according to the same method. Mice were infected intraperitoneally by a suitable dose of pneumococci from a 24 hours' old culture on 5% blood broth medium. The treatment was started one hour after the infection. Preparations were given (by gastric sound) thrice daily for six or seven days. Of every animal that died during the period of observation (10 days), microscopic preparations of the spleen were tested for pneumococci. When these were absent, the animal was discarded.

Spontaneous variations in the pneumococci strains (no R—S variations) were a frequent source of failure; in these cases the administered dose of

Table 2.

Time of observation 7 days.

preparation	daily dose in mg	number of animals	dead on							survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day		
controls	—	6	1	4	1	—	—	—	—	—	2.0
sulfanilamide (per os)	20	6	—	—	4	2	—	—	—	—	3.3
sulfanilamide . . . . + pyridine (subcut)	20 + 5	15	—	—	1	4	6	1	—	3	5.0
sulfanilamide . . . . + pyridine-hydrochloride (per os) . .	20 + 7	9	—	—	—	2	4	1	—	2	5.0
sulfapyridine (per os)	20	4	—	—	—	—	—	—	—	4	7.0

sulfanilamide cured the animals, or retarded their death, in a way that rendered the readings confusing.

The results are given in condensed form, some typical tables (see tables 3, 4, 5 and 6) having been selected. Nearly all experiments were repeated several times.

All substances other than sulfanilamide and sulfapyridine, when administered alone, were inactive.

Several experiments (out of which table 5 is a specimen) showed us either the possibility of an overdose of the supplementary drug, or at all events the existence of an optimum. So the pyridine compound should form a merely small part of the total.

If this holds true with regard to sulfapyridine itself as well, it should be possible to substitute a part of the just effective dose of sulfapyridine by sulfanilamide (table 7 and 8).

The latter experiments again show that sulfapyridine must be looked upon as a substance with two different faculties:

a. it acts in quite the same way as sulfanilamide,

b. as a pyridine derivative, it has a supplementary activity embodied in the molecule. This additional faculty can be exerted by pyridine derivatives totally devoid — when administered singly — of antibacterial activity.

So far, only pyridine compounds that are more or less related to sulfapyridine have been considered. But pyridine derivatives are anything but uncommon in pharmacology. We tested chinine, which is widely used and can be considered as an α-substituted pyridine substance. It was administered, singly and in combination with sulfanilamide, to mice infected with pneumococci. Unfortunately, mice being rather sensitive to chinine, it could merely be given in small doses. Some typical experiments are recorded in tables 9, 10 and 11. The combination of sulfanilamide with some chinine compounds has, though starting from quite another idea, been realised before (3).

We finally tried some unrelated substances: the heterocyclic substance pyramidon, also acetanilide (antifebrine), para-amido-phenol, aceto-salicylic acid, and thiophen. It is unnecessary to give all protocols: thiophen and para-amido-phenol cannot improve the therapeutic effect of sulfanilamide. Aceto-salicylic acid has a slight supplementary action. Pyramidon is fairly active (see table 12).

Acetanilide, however, proved to be one of the best among the substances tested by us (see table 13), its action being comparable to that of chinine. (The action of thiazol-compounds could not yet be studied).

Table 3.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	6	—	6	—	—	—	—	—	—	—	—	—	2.0
sulfanilamide . . .	20	6	—	—	2	4	—	—	—	—	—	—	—	3.7
sulfanilamide . . . + dibenzoyl-amido-pyridine	20 + 10	6	—	—	1	2	1	—	—	—	—	1	1	7.4
sulfanilamide . . . + monoacetyl-amido-pyridine	20 + 6	6	—	1	—	3	1	1	—	—	—	—	—	4.1

Table 4.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	6	6	—	—	—	—	—	—	—	—	—	—	1.0
sulfanilamide . . .	20	6	—	—	2	3	1	—	—	—	—	—	—	3.8
sulfanilamide . . . + potassium methyl-α-amino-pyridine	20 + 5	6	—	—	1	1	2	—	—	—	—	—	2	6.1
sulfapyridine . . .	20	6	—	—	—	—	—	—	—	—	—	—	6	10.0

Table 5.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	4	1	3	—	—	—	—	—	—	—	—	—	1.8
sulfanilamide . . .	20	4	—	—	—	—	3	—	1	—	—	—	—	5.5
sulfanilamide . . . + α-benzene-sulfonamido-pyridine .	20 + 5	6	—	—	—	—	—	—	4	1	—	—	1	7.4
sulfanilamide . . . + α-benzene-sulfonamido-pyridine .	20 + 10	6	—	1	—	1	1	1	1	1	—	—	—	5.3
sulfapyridine . . .	20	6	—	—	—	—	—	—	—	1	—	—	5	8.6

Table 6.

preparation	daily dose in mg	number of aniamls	dead. on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	4	—	4	—	—	—	—	—	—	—	—	—	2.0
sulfanilamide . . .	20	6	—	—	1	2	3	—	—	—	—	—	—	4.3
sulfanilamide . . . + toluene-sulfonamido-pyridine	20 + 5	6	—	—	—	—	—	—	—	1	—	—	5	9.7
sulfapyridine . . .	20	6	—	—	—	—	—	—	—	—	—	—	6	10.0

Table 7.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	6	6	—	—	—	—	—	—	—	—	—	—	1.0
sulfanilamide . . .	20	6	—	—	2	3	1	—	—	—	—	—	—	3.8
sulfanilamide . . . + sulfapyridine . . .	16.3 + 5	6	—	—	—	—	3	—	—	—	1	—	2	7.3
sulfapyridine . . .	5	6	—	—	2	2	2	1	1	—	—	—	—	4.1
sulfapyridine . . .	20	6	—	—	—	—	—	—	—	—	—	—	6	10.0

Table 8.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	6	—	6	—	—	—	—	—	—	—	—	—	2.0
sulfanilamide . . .	20	6	—	—	2	1	1	1	—	—	1	—	—	5.0
sulfanilamide . . . + sulfapyridine . . .	15 + 7.3	6	—	—	—	—	2	—	—	—	—	—	4	8.3
sulfapyridine . . .	7.3	6	1	3	1	—	—	—	—	—	—	—	1	3.7
sulfapyridine . . .	20	6	—	—	—	—	—	—	—	—	—	—	6	10.0

Table 9.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	6	—	6	—	—	—	—	—	—	—	—	—	2.0
sulfanilamide . . .	20	6	—	—	2	4	—	—	—	—	—	—	—	3.7
sulfanilamide . . . + chinine (hydrochloride)	30 + 10	15	—	—	—	—	1	—	2	3	—	3	6	9.3

Table 10.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	6	—	4	2	—	—	—	—	—	—	—	—	2.3
sulfanilamide . . .	30	6	—	—	—	—	2	3	—	—	—	—	1	6.3
sulfanilamide . . . + chinine (hydrochloride)	30 + 10	6	—	—	—	—	1	1	1	—	—	—	3	8.0

Table 11.

preparation	daily dose in mg	number of animals	dead on 1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day	survivals	mean survival (days)
controls	—	6	6	—	—	—	—	—	—	—	—	—	—	1.0
sulfanilamide . . .	20	6	—	—	2	3	1	—	—	—	—	—	—	3.8
sulfanilamide . . . + chinine	20 + 5	6	—	—	—	2	1	1	—	—	—	—	2	6.5
sulfapyridine . . .	20	6	—	—	—	—	—	—	—	—	—	—	6	10.0

Table 12.

preparation	daily dose in mg	number of animals	dead on 1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day	survivals	mean survival (days)
controls	—	6	—	6	—	—	—	—	—	—	—	—	—	2.0
sulfanilamide . . .	20	6	—	—	—	6	—	—	—	—	—	—	—	4.0
sulfanilamide . . . + pyramidon	20 + 5	6	—	—	—	—	3	1	1	—	—	—	1	6.3
sulfapyridine . . .	20	6	—	—	—	—	—	—	—	—	—	—	6	10.0

Table 13.

preparation	daily dose in mg	number of animals	dead on										survivals	mean survival (days)
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day		
controls	—	4	—	4	—	—	—	—	—	—	—	—	—	2.0
sulfanilamide . . .	20	6	—	—	3	1	1	1	—	—	—	—	—	4.0
sulfanilamide . . . + acetanilide	20 + 10	5	—	—	—	1	1	—	1	—	—	—	2	7.4

Summary and Comments.

With the object of investigating the nature of the remarcable difference in therapeutic effect between sulfanilamide and sulfapyridine we based ourselves on the assumption that the latter substance has two different actions combined in its molecule, *i.e.* the action of sulfanilamide and the action of a pyridine derivative. Experiments in vitro (not published here) have indicated that the first supposition holds true; the second assumption is dealt with in this paper.

We have tried to imitate the action of sulfapyridine by administering — simultaneously with sulfanilamide — various substances to animals infected with such a strain of pneumococci that sulfapyridine might cure, not, however, sulfanilamide. We tried pyridine, which improved, though only slightly, the action of sulfanilamide, pyridine itself being inactive as a therapeutic. Step by step we tried pyridine derivatives, finally building up toluene-sulfon-amino-pyridine, *i.e.* sulfapyridine of which the NH_2 group is substituted by CH_3. This substance is quite inactive as a therapeutic, but combined with sulfanilamide it greatly enhances the latter's effect. So we actually may conclude that the action proper to sulfapyridine (Dagénan, M & B 693), as compared to the mother substance sulfanilamide, must be attributed to the fact that another activity, next to that of sulfanilamide, resides in the molecule.

Other drugs may have a similar effect. Chinine hydrochloride and acetanilide, added to sulfanilamide, also greatly improve its therapeutic effect, although our experiments indicate that the action of sulfapyridine is not equalled.

As far as we know, this is the first attempt to explain the therapeutical progress embodied in sulfapyridine. The facts, given above, however, should only be considered as a beginning. The mechanism of the supplementary action is still unknown. There is some evidence of its acting on the body and not on the bacteria, but this point of view may be open to revision. In our opinion this new consideration is not merely of theoretical value.

The original sulfanilamide drug has unmistakable advantages. Maybe its imperfections can be corrected by the mere additional administration of some very common drugs, which would mean already some gain. The possibility, however, of finding drugs that might supplement the action of sulfanilamide in the case of infections where this kind of therapy had failed or proved unsatisfactory, seems to be of even greater importance.

The number of possibilities, however, is so confusingly large, that we thought it be best to publish the results we obtained.

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(From the Laboratory of the „Keuringsdienst van Waren", Rotterdam).

A REVISION OF THE PROCEDURE FOR THE VOGES-PROSKAUER TEST

by

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(Received June 3, 1941).

Although other well-known reactions for the detection of diacetyl are in existence, apparently the procedure for the Voges-Proskauer test as described in the Manual of Methods for Pure Culture Study of Bacteria (Cf. Leaflet V_{39}, 17—18) is always returned to. This procedure consists in an incubation at 37° C. during 5 days in the methyl red medium — 5 g peptone, 5 g glucose, 5 g K_2HPO_4 in 1000 ml distilled water —; after addition of an equal amount of a 10% solution of KOH in a culture tube the solution is kept for another day. A positive Voges-Proskauer reaction is indicated by an eosin color.

In our laboratory the following revised method is in use. A pure strain of the coli group is inoculated into a test tube provided with about 10 ml of the following medium: 2% glucose, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% NH_4Cl (or $(NH_4)_2SO_4$) and 1% $CaCO_3$. This culture is incubated for 24 (or if necessary for 48) hours at 37° C. To three drops of the culture solution in a test tube three drops of 4 n NaOH solution and one drop of a 1% creatine solution are added. The mixture is placed in a water bath at 45—50° C. and is shaken now and then. In case of a positive reaction the red colour will show in one or two minutes.

The usual method for the Voges-Proskauer test has been revised for two reasons. Firstly the methyl red medium gives only a weak reaction, because it is too low in content of glucose and creatine. Secondly the more or less yellow shade of the peptone disturbs the recognising of a red colour.

The revised method allows a vigorous culture of *B. aerogenes* to show the reaction after two or three days' incubation even when diluted 1 : 20.

The creatine solution may be conserved by adding some drops of toluene.

(From the Laboratories of the City Health Department, Amsterdam).

ON THE BEHAVIOUR OF *E. TYPHI* IN SURFACE WATER

by

A. CHARLOTTE RUYS

(Received June 11, 1941).

Since Wilson and Blair published their glucose-bismuth-citrate-fosfate medium (14) we are able to isolate typhoid bacteria from contaminated surface water and to study the behaviour of these microbes under various environmental conditions.

In our epidemiological investigations on typhoid infections in the neighbourhood of Amsterdam we were struck by the fact, that especially in winter months, it often was contaminated water, which was the source of typhoid infections.

So in the village of Uithoorn on two occasions during the month of February we isolated typhoid bacteria from a ditch which had been infected by the feces of a typhoid patient (6). Typhoid bacteria were cultivated from the water of the ditch even 24 days after the sewage from the patients house had flown into the ditch for the last time. Milk infected by the contaminated water was the source of infection in three other cases of typhoid fever.

In the very cold winter of 1940 we found typhoid bacteria in a piece of ice from a canal on which children had been skating. One of them was thought to have infected herself with typhoid bacteria by eating a piece of ice to quench her thirst.

In October 1940 we could trace a case of typhoid fever to the eating of nuts which had fallen into a ditch contaminated by the non purified sewage from a few houses in one of which lived a chronic carrier of typhoid bacteria. Before the carrier had been detected *E. typhi* was isolated from the ditch several times during the winter 1940/41 (8).

Most of our investigations however were carried out in the small town of Volendam (7, 10), where we isolated for the first time typhoid bacteria in March 1936. A child suffering from typhoid fever most probably infected herself when playing with water from a canal near her home. Indeed we found this water most heavily contaminated and also (but to a lesser extent) the water at several places at a distance of some hundred meters.

Table 1 gives the results of a series of investigations carried out during 1936—1939. It shows that, apart from the heavy contamination during the spring of 1936, only in winter months typhoid bacteria could be detected in the water.

Table 1.

Typhoid bacteria in Volendam canals.

Date	*E. typhi*	Eijkman titer	Date	*E. typhi*	Eijkman titer
1936 26/3	+	0.0001	5/10	—	0.001
1/4	+++	0.001	3/11	—	0.001
14/4	++	0.01	1937 18/1	++	
6/5	++	0.001	2/2	—	0.001
9/5	++	0.0001	23/3	—	0.1
19/5	+	0.01	11/5	—	0.0001
26/5	+	0.01	3/8	— 1)	0.0001
2/6	++		5/11	+	
3/6	+	0.0001	15/11	++	0.001
17/6	—	0.1	1938 6/4	—	0.1
1/7	— 1)	0.0001	11/4	—	
29/7	— 1)	0.001	20/4	—	0.01
25/8	— 1)	0.001	9/11	++	0.001
29/9	—	0.01	1939 20/2	—	0.01

1) overgrown with black colonies of bacteria from the colon group.
+ = one colony in 5 ml.
++ = a few colonies in 5 ml.
+++ = more than 20 colonies in 5 ml.

The Eijkman test indicates that the water is heavily polluted with fecal material all over the year. In July and August the water may contain so many bacteria growing in black colonies that it sometimes is practically impossible to isolate a single typhoid colony among them. Houston (4) also drew attention to this fact. In other summer months we did not encounter these difficulties.

The source of the infection with typhoid bacteria could not be detected. It is supposed that during springtime 1936 the sewage from a carrier living near the home of the patient was deposited into the canal and that the carrier left the place afterwards. The feces of the patient were all thoroughly desinfected and there were no other patients in the neighbourhood. As typhoid fever has been endemic in Volendam during many years it is not surprising that there should be several carriers and that the heavy fecal pollution throughout the whole town regularly should bring typhoid bacteria into the water. The only fact, which might be wondered at was the frequency of positive findings in winter months, next to its absence in the summer, typhoid fever having its maximum incidence during the late summer.

I therefore tried to get more information about the behaviour of typhoid bacteria in surface water, from which they are known to disappear after a more or less extended period.

In older literature the self purification of the water is ascribed to the action of the light, partly to the direct sunlight and to a lesser extent to the diffuse daylight (see ROTHERMUNDT (9), WIESNER (13) and BUCHNER (1)).

In later publications the protozoa have been introduced as the most effective factor in the process of water purification and subsequently very little attention has been paid to the action of the light. In many experiments it was even entirely disregarded.

HOUSTON laid much stress on the influence of the temperature (3). He stated, that typhoid bacteria would disappear from raw river water much more rapidly the weather being warm than when it was cool.

Other investigators pointed out, that the decrease in number of bacteria in surface water may also be due to harmful chemicals. So JONGBLOED (5) stated that in surface water sterilised by filtration the chemical composition determines the lifetime of *E. coli*. When he composed a water with chemicals in quantities such as were found in raw canal water he observed the same extinction line of *E. coli* as in the natural water.

WIBAUT-ISEBREE MOENS (12, 13) observed that typhoid bacteria vanished from water of a ditch very rich in microbes only slightly more rapidly than from the same water which had been filtered before inoculation with the typhoid bacteria. So besides the protozoa other factors must influence the disappearance of *E. typhi* from the water.

As WILSON and BLAIRS medium now renders experiments with typhoid bacteria under natural conditions much more reliable than with the methods such as they were used before, I tried the following experiments.

Primarily some samples of naturally infected Volendam water were put in the incubator at 24° C. and in the ice box at 6° C. both in the dark. The typhoid bacteria lived longer in the cold than at 24° C. In some experiments at 24° C. an overwhelming growth of bacteria of the colon group blackened the WILSON and BLAIR plates and made the isolation of the scantily occurring typhoid bacteria impossible. In other samples there was no multiplication of bacteria and the typhoid bacteria died of very quickly, always within 6 days. When stored in the cold they were always longer living. As a maximum we found 11 days. According to the EIJKMAN test *E. coli* also died of much more rapidly at 24° C. than at 6° C.

Then surface water from various canals, ditches and ponds were partly sterilised by filtration through a Chamberland filter or a Seitz filter which had been plated with chromium in order to avoid the oligodynamic action of the metals of the ordinary Seitz filter. The raw water and the filtrate both were inoculated with a not over large amount of typhoid bacteria and put outside the laboratory in diffuse daylight in Jena flasks in quantities of 500 ml. Some of the flasks were envelopped with a thin dark cloth in order to avoid the influence of the light. In some experiments protozoa cultivated on dead *E. coli* were added to the filtered water (Protozoa are known to be as active in the dark as in the light). All these experiments gave the same result. Typhoid bacteria vanished rapidly from the water and the decrease in number of the typhoid bacteria was more influenced by the diffuse daylight than by the action of protozoa.

The results were approximately the same with raw water and with filtrate to which protozoa had been added (table 2). So the disappearance of typhoid bacteria is not materially influenced by the presence of other bacteria.

Only in one out of a large series of experiments we found but small difference between the samples kept in the dark and those in the light. It was in November 1938 when it took more than 5 days before the action of the light could be demonstrated. After 12 days however the difference was striking.

In some experiments there were even more typhoid bacteria in the samples with protozoa than in those without. The only difference between the two series is that a rather large quantity of organic matter is added to the filtrate along with the cultures of the protozoa.

We made some further experiments with *E. coli* in order to ascertain whether these microbes are influenced by the light to the same extent as *E. typhi*. In judging the hygienic condition of a surface water it is not *E. typhi* which is sought for, but the amount of *E. coli* which is deter-

Table 2.

Material	typhoid bacteria per ml	temperature	date	number of typhoid colonies per ml			
				in diffuse daylight		in the dark	
				after 3 days	after 5 days	after 3 days	after 5 days
1. River water (Amstel)			1938				
a. raw	7000	7—12° C.	May	200	0	800	31
b. filtrate	7000	7—12° C.	May	500	0	4200	1100
c. filtrate + protozoa	7000	7—12° C.	May	1600	1	5000	45
2. River water (Amstel)			1941				
b. filtrate	8500	3—8° C.	March	370	7	2175	830
c. filtrate + protozoa	8500	3—8° C.	March	540	23	3190	4100
				after 2 days	after 4 days	after 2 days	after 4 days
3. Pond water (Oosterpark)			1939				
a. raw	5000	4—7° C.	March	102	2	1240	70
b. filtrate	5000	4—7° C.	March	40	0	680	15
c. filtrate + protozoa	5000	4—7° C.	March	32	0	430	18
4. River water (Amstel)			1938				
a. raw	4500	12—22° C.	May	100	0	600	0
b. filtrate	4500	12—22° C.	May	700	0	4300	0
c. filtrate + protozoa	4500	12—22° C.	May	100	0	1400	0

mined. So for hygienic purposes it is necessary to know also the sensitiveness of *E. coli*.

Experiments were made with raw river water, filtrates and filtrates which had been provided with a culture of protozoa. To economise agar which is now difficult to obtain, I used in the tests with *E. coli* the silicate medium of HETTCHE and MÜNCH (2), on which they grow in yellow colonies.

A comparison of the series of experiments made with both microbes showed that *E. coli* is more resistent to diffuse daylight than *E. typhi*.

We nearly always noted an initial increase in number of *E. coli* followed by a more or less rapid decrease.

Only when a very large amount of protozoa was added and the temperature was not too low, there was no initial increase. But when during a few days the light was exceptionally bright *E. coli* as well suffered severely from the action of the light, its action being then more pronounced than that of the protozoa.

Up till then all the investigations were made in surface water in which very little food was available for *E. typhi* and perhaps somewhat more for the less exacting *E. coli*. Therefore also some experiments were made under more favourable nutritional conditions with broth inoculated with fresh cultures of either *E. typhi* or *E. coli*. The behaviour of both organisms was studied in samples of 75 ml in Jena flasks which were put outside the laboratory, one exposed to the diffuse daylight and one kept in the dark. Another one was put in the ice box at approximately 4° C. The temperature in the open varied between 1° and 12° C. Whilst the number of typhoid bacteria increased out of doors in the dark during the first days, they immediately made a rapid decrease in the light. In the ice box the number also diminished but more slowly. *E. coli* contrarily suffered very little from the light, which only impeded the multiplication to some degree (see table 3).

Table 3.

Date	*E. typhi* in broth 2/3/'41 initial count 26.000 per ml			*E. coli* in broth 2/3/'41 initial count 260.000 per ml		
	outside 1—12° C.		ice box 4° C.	outside 1—12 C°.		ice box 4° C.
	dark	light	dark	dark	light	dark
3/3/'41	38.800	1080	4800	10.000.000	3.700.000	500.000
4/3/'41	34.100	40	4950	16.600.000	5.500.000	490.000
6/3/'41	15.500	1	3800	40.000.000	6.250.000	280.000

When however the weather was warmer the multiplication of the typhoid bacteria was stimulated to such a degree that the influence of the light could be neglected. In experiments made at temperatures varying between 9° and 17° C., the bacterial count augmented as rapidly in the light as in the dark. So we may conclude from these experiments that typhoid bacteria are killed by the action of diffuse daylight if they live under conditions in which they are not able to multiply rapidly. These unfavourable conditions are realised in surface water and in broth at low temperature.

I made no experiments to find out which part of the spectrum is most active. As the above related experiments were made in flasks of Jena glass which is impervious for ultraviolet rays, the typhoid bacteria must be sensitive for other parts of the spectrum too. A few experiments were made with open glass vessels (17 cm diam.) and the same vessels covered with a thin piece of glass. There was no significant difference in the bacterial count of both.

The significance of the action of the light for the self-purification of surface water under natural conditions however depends on the behaviour of typhoid bacteria towards the light, *i.e.* whether they are attracted by the light or whether they flee it.

In the older literature opinion is at variance about this question. ROTHERMUNDT says that river water-bacteria flee the light, whilst in WIESNERS opinion there is a definite phototaxis. WIESNER compared the bacterial counts of both ends of a U formed tube filled with a typhoid culture broth one end of which had been wrapped in dark paper, with the counts from two similar tubes, one kept entirely in the light, the other one in the dark. After exposure to light for some time in either end of the semi covered tube a nearly equal amount of typhoid bacteria were found, *viz.*, in both slightly less than the initial count. In the dark tube there had been a rapid increase and in the light one a decrease could be noted. These results can only be explained by a moving of typhoid bacteria to the illuminated side of the semi covered tube.

I repeated these experiments with some minor changes and I was able to confirm the results with the restriction that they had to be performed in fairly cool weather (4°—8° C.). When however the outdoor temperature was higher the typhoid bacteria were able to multiply in the broth in spite of the influence of the diffuse light. The temperature varying between 9° and 17° C. there occurred no appreciable difference between the tubes kept in the diffuse light and those in the dark. After 3 days the initial bacterial count had risen from 65.000 per ml to 820.000 in the tube entirely exposed to the light, to 950.000 in the one entirely kept in the dark and to 820.000 and 670.000 per ml in the light and the darkened side of the semi covered tube.

In experiments made with raw river water contaminated with typhoid bacteria in U form tubes at temperatures between 5° and 18° C. the results were not so clear-cut.

Sometimes after one day's exposure the dark end of the semi covered tube contained slightly more bacteria than the wholly dark tube. But after 3 days the results were always the same: no more bacteria in the light tube, the maximum in the dark one, a few in the light end and some more in the dark one of the semi covered tube. So in the beginning there may have been an indication of photophobia but afterwards a definite phototaxis must have occurred.

In order to approach more nearly the natural conditions in ditches I then made a series of experiments in a stone jar, 50 cm high, 30 cm diameter, which was filled to the top with raw river water mixed intensely with a suspension of freshly cultured typhoid bacteria. A small stone jar and a glass one both 17 cm in diameter were filled only to a height of 4 cm with some of the same water. The stone jars were put in the open in diffuse daylight, the glass one at the same place but in the dark.

The temperature varied between 5° and 19° C. From the large jar samples were taken at the surface and at the bottom separately. From the smaller jars the water was taken after having been thoroughly mixed.

After an exposure for one or two days the difference was striking. In the small jar in the light the decrease had been most pronounced. The water from the surface layer of the large jar always contained many more typhoid bacteria, which fact in my opinion only can be explained by a constant flow of typhoid bacteria from the bottom to the top. In accordance with this surmisal was the fact that typhoid bacteria had disappeared always more rapidly from the bottom of the large jar than from the narrow layer of the dark jar (see table 4). In a series of 6 experiments in these jars one showed an irregularity which we could not account for. In this experiment the typhoid bacteria vanished more rapidly from the large jar (bottom and surface) than from the small jar in the light, whilst the protozoal count was much larger in the small one.

During the short period of observation in these experiments colon bacteria diminished very slightly as was shown by the Eijkman test. There were some minor variations in the Eijkman titer but they were so irregular that I was unable to draw any conclusion from them.

The experiments were made with an old laboratory strain and with two freshly isolated ones. We could not state a difference of sensibility against the various noxious influences. There were minor variations in the rapidity with which the bacterial count diminished but this is not surprising, neither the chemical constitution of the water nor the

Table 4.

Material	Date	Water kept in diffuse daylight			Water kept in the dark
		jar diameter 30 cm water column 45 cm number of typhoid colonies per ml water		jar diameter 17 cm water column 4 cm number of typhoid colonies per ml water	jar diameter 17 cm water column 4 cm number of typhoid colonies per ml water
		surface	bottom	mixed	mixed
1. Raw Amstel water 1)	1/4	38.000	38.000	38.000	38.000
	2/4	1.280	7.130	370	11.000
	3/4	430	1.030	2	6.000
	4/4	2	19	0	60
	5/4	0	8	0	6
2. Raw Amstel water 2)	22/4	35.000	35.000	35.000	35.000
	23/4	11.500	11.100	13.700	11.300
	24/4	9.000	6.800	2.000	4.900
	25/4	1.900	3.800	280	3.500
	26/4	750	800	18	1.650
	28/4	11	27	0	110
3. Raw pond water 3) (Oosterpark)	7/5	45.000	45.000	45.000	45.000
	8/5	9.700	11.000	2.800	8.700
	9/5	450	540	0	925
	10/5	18	7	0	300
	11/5	0	4	0	160
4. Raw pond water 4) (Oosterpark)	13/5	76.000	76.000	76.000	76.000
	14/5	24.000	33.000	6.250	45.000
	15/5	17.500	15.900	154	32.000
	16/5	3.800	3.000	1	16.000
	17/5	350	230	0	2.600

1) with 370 mg Cl per l, $KMnO_4$ 101, turbid. Mixed with typhoid bacteria (H 901). Temperature between 3° and 6° C., weather half cloudy, half sunny.

2) with 483 mg Cl per l, $KMnO_4$ 65, turbid. Mixed with typhoid bacteria (1199). Temperature between 6° and 10° C., on 25/4 and 27/4 sunny weather, the other days cloudy.

3) with 170 mg Cl per l, $KMnO_4$ 95, perfectly clear. Mixed with typhoid bacteria (H 901). Temperature between 11° and 16° C., weather half sunny, half cloudy.

4) with 180 mg Cl per l, $KMnO_4$ 125, perfectly clear. Mixed with typhoid bacteria (Kok). Temperature between 12° and 18° C., weather half cloudy, half sunny.

metereological conditions being quite the same. No bacteriophage active against typhoid bacteria was found in the water.

Discussion.

The observations made under natural conditions related above indicate that the positive finding of typhoid bacteria in surface water was more frequent in the colder months than in the warm period. Laboratory experiments confirm that the lifetime of typhoid bacteria in surface water must be longer during winter than in the summer. In summer not only the higher temperature destroys the typhoid bacteria in water more rapidly, but also the protozoa are more active and the light is more intense and acts during a much longer period. The laboratory experiments indicate that the disappearance of typhoid bacteria is accelerated to a high degree by the influence of the light. Practically always the diffuse daylight was more active than the protozoa. Most probably in naturally infected water the protozoa will not distinguish between typhoid and other bacteria. The light however does much more damage to the typhoid bacteria than to the bacteria of the colon group. So the supposition that from the hygienic point of view the light is more effective in the purification of contaminated water is quite acceptable.

The top of the frequency of typhoid fever in man always lies in the late summer months, in a period in which the lifetime of typhoid bacteria in water is short.

Perhaps the much more frequent contact with water during the summer causes the increase in typhoid infections in spite of the rapid disappearance of the microbe from the water.

Summary.

1. Several typhoid infections were traced to direct or indirect contact with contaminated water from which typhoid bacteria could be isolated.
2. Especially during the winter months typhoid bacteria could be isolated from surface water.
3. In experiments the disappearance of typhoid bacteria from contaminated water is accelerated much more by the influence of the light than by the action of protozoa.

4. The definite phototaxis of typhoid bacteria may cause the rapid decrease of the microbes from the depth of surface water as well.

I wish to express my thanks to Dr. N. L. Wibaut-Isebree Moens and to Dr. A. Jaarsveld for their most valuable help, especially in the experiments with protozoa.

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(From the Laboratory of the Waterworks, Rotterdam).

ON THE DISAPPEARANCE OF *B. COLI* AND FAECAL STREPTOCOCCI (ENTEROCOCCI) AS THE RESULT OF SLOW SAND FILTRATION

by

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Before discussing this subject it may be useful to describe shortly the process of purification of river water for the obtaining of drinking water, so as it is applied at Rotterdam.

The filterplant of the Waterworks is provided with two long, narrow basins, in which the water is left to plain sedimentation and which are alternately in service. Nowadays the river water from the river Rhine (in the neighbourhood of Rotterdam called Nieuwe Maas) is pumped only at low tide into an intake canal and flows through sluices into the sedimentation basin, not in actual service at that moment. The retention period therefore lasts from ten to twelve hours. The pumping of the river water into the basins was considered an improvement to the direct inflow, such as it was in use till the middle of 1939, because at high tide the sewage of the city of Rotterdam, although very diluted, could reach the intake canal.

After sedimentation the water is pumped to the prefilters of the open (gravity) type Paterson (London). As the mere rapid sand filtration does not provide for a sufficient removal of bacteria, no coagulation being practised, prefiltration is followed by a slow sand filtration. After the installation of the primary filters the rates of filtration through the secondary beds (slow sand filters) could be considerably increased without involving any trouble.

Prefiltration is carried out at a rate of 4—5 m^3 per hour; during summer this rate may be accelerated to 6—7 m^3 per hour, without injuring the effect. Prefiltration takes place through a layer of 75 cm of coarse sand (size 0.5—2 mm) borne by layers of gravel. The prefiltered water passes through a double water channel to the slow sand filters. Slow sand filtration is practised at a rate of 5—15 cm (sometimes during summer 20 cm) per hour through a sand layer of 1.5 m, also resting on a layer of gravel. This layer again is borne by bricks, arranged in a way so as to form narrow channels at the floor level which facilitate the withdrawal of the water. The openings between the bricks permit a uniform rate of percolation through the filtering medium. This type of sand filter is known on the continent as the ,,English'' system. The sand of the slow sand filters is graded in accordance with predetermined standards. The Hazen formulae for effective size and uniformity coëfficient are as a matter of course taken into account in the composition of the two filter materials. The slow sand filtered water is led by two pure water channels I and II, then chlorinated for sterilisation and pumped to the town. In winter (middle of November till middle of May) activated carbon for the elimination of any undesirable taste or odor is dosed in a quantity varying from 3—7 mg per l to the settled water just before prefiltration. Samples are collected from the prefiltered water and from the water of the two pure channels at definite points.

The two prefiltered water samples are mixed in equal amounts before bacteriological examination for Streptococci and for *B. coli* takes place. The same holds for the two pure water samples.

To detect *B. coli* and faecal Streptococci the enrichment methods published in a former paper (5) are used. The fermenting flasks with the glutamic acid medium and the positive indole tests in the bacto-tryptone Na-formate medium are streaked over the surface of Levine agar plates after 24 and 48 hours' incubation at 44.5° C; these plates are examined after 20—24 hours' incubation at 37° C.

As indole production by bacteria has been shown to depend upon the presence of the tryptophane group, bacto-tryptone is exceptionally well suited for use in the indole test, because it is rich in this nitrogen compound.

The presence of long-chained Streptococci (12—50, sometimes 200 and more cocci) is easily detected by microscopical examination. To meet purposes of isolation, for the peptone-glucose-caffein-medium, the usual meat-extract-gelatine or agar plus 0.5% glucose may be used. When however lactose-peptone has been used as enrichment medium, which is much less selective, isolation on agar containing 0.5% lactose, 1% peptone, 0.5% NaCl, 0.2% K_2HPO_4 with the addition of potassium tellurite 1 : 15000, as recommended by HAROLD (6) is to be preferred.

The results of the bacteriological examination for the years 1938, 1939 and 1940 are shown in figure 1, where the coli and streptococci titers of the prefiltered and slow sand filtered water are represented. As regards the temperature of the slow sand filtered water, the averages of three daily observations are recorded. The water level of the river Nieuwe Maas is recorded in M + R.P. (level at Rotterdam).

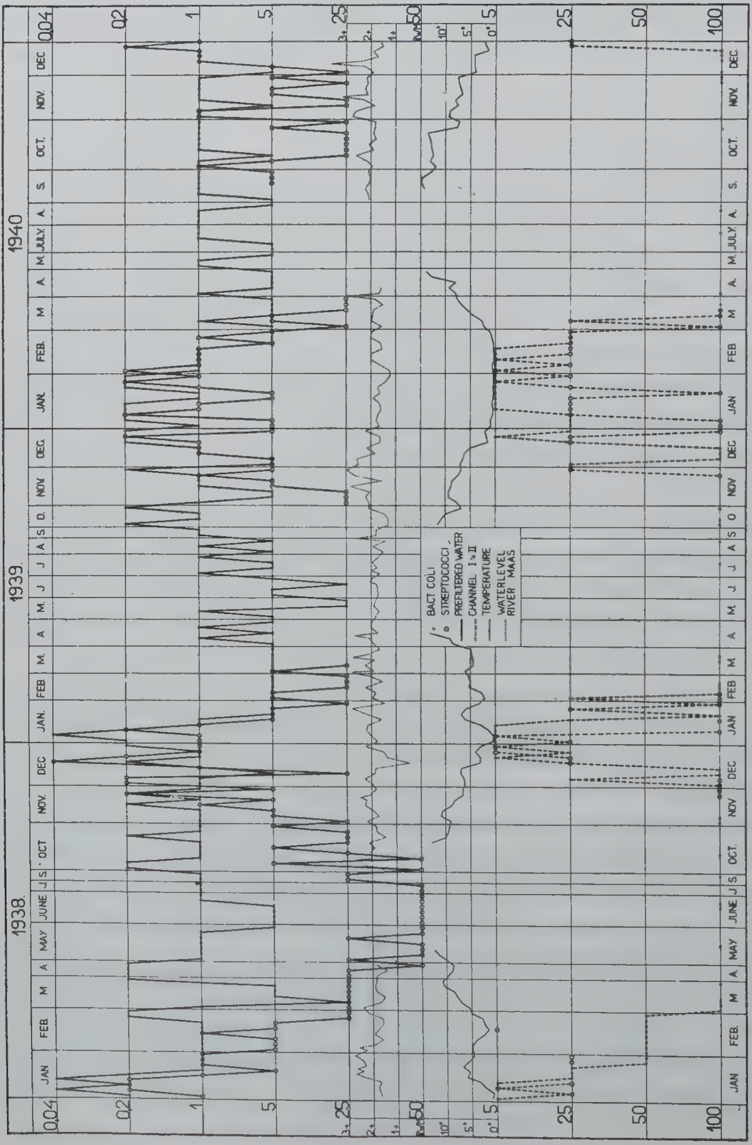

Fig. 1.

From this figure the following conclusions may be derived:

1°. Faecal Streptococci only appear in the secondary filtrate (slow sand filtered water) when the temperature of the water is low, *viz.* in the end of November and during December, January and February. In exceptionally severe winters they may still occur as late as the middle or end of March.

2°. In small quantities of slow sand filtered water, *viz.* 5 ml, faecal Streptococci may be found only during a very severe frost period, when the temperature of the water under the ice deck sinks below 1° C.

3°. They disappear from the slow sand filtered water in the latter part of February in normal winters; in severe winters, however, (1939—1940) in the latter half of March, when the temperature of the water has risen to 2—3° C., *viz.* as soon as the filters are free from ice.

4°. In prefiltered river water (primary filtrate) faecal Streptococci occur in definite amounts of water, *viz.* 0.2, 1, 5 or 25 ml, according to temperature in all months of the year with the exception of April, May, June, July, August and the greater part of September. In the year 1938, however, they were often found in these months in 25—50 ml; in fact in that year the river water was merely led through a simple inflow into the sedimentation basins for want of a pumping equipment. In September and October 1939 no tests for Streptococci could be made.

From April up to August often not any Streptococci may be detected in samples as large as 100 ml of prefiltered water. This is not merely due to prefiltration; in fact in the settled water of the sedimentation basin the Streptococci disappear at a high rate. Next to the letal effect of the sunlight and the natural death rate they are very rapidly ingested and destroyed by Amoebae and other protozoa (1, 2, 7, 8, 10, 11, 12, 14). Louwe Kooymans (9) mentions already experiments with pure cultures of the Ciliates *Uronema nigricans, Lembus pusillus* and the Flagellates *Monas vulgaris, Bodo mutabilis* and *Pleuromonas jaculans,* isolated by cultivation on faecal Streptococci.

As a demonstration of their rapid disappearance in the sedimentation basins may serve an estimation on April 9th, 1941 of the titer for Streptococci of the inflowing water at low tide in one of the sedimentation basins which was 1 ml, whilst this titer after 12 hours' settling appeared to be 25 ml. In contrast to their rapid disappearance in this period stands their occurrance in the period from the end of September to the end of March. Along with the decrease in temperature of the water the number of Streptococci increases. They occur in largest numbers when the sedimentation basins as well as the sand filters are covered with an ice deck. The explanation of this fact consists forcibly in the encysting of the protozoa as a result of the low temperature of the water, by which means the natural enemies of the Streptococci are eliminated.

The poorer purification of the water by slow sand filtration at periods of severe frost is not only indicated by the appearance of faecal Streptococci into the secondary filtrate, but also by a partly inhibition of the nitrification. In fact the longer the frost remains the larger are the amounts of ammonia nitrogen occurring in the secondary filtrate, whilst albuminoid- and nitrite nitrogen occur in larger amounts than usual as well. The oxygen consuming capacity increases such as the number of bacteria occurring on nutrient gelatin and agar. As soon as the frost is over the protozoa decyst and destroy the Streptococci.

On a laboratory scale this process may be reproduced by the following experiment. A number of Erlenmeyer flasks are filled with river water. To each of them faecal Streptococci, grown from a single colony in a suitable culture medium and washed several times with distilled water until free from the adhering medium, are added in a quantity so as to render the water slightly opalescent. After a period of six to eight days, varying with temperature, the cultures begin to clear up and the presence of many protozoa (Ciliates, Flagellates, Amoebae) is readily stated by microscopical examination. If, however, the flasks with the faecal Streptococci are kept in a refrigerator at 2° C. no clarifying of the opalescent cultures follows; even an initial clearing up is brought to a standstill. The same prevails with cultures of *B. coli*.

LOUWE KOOYMANS dit not succeed in cultivating a phage which would act on Streptococci. Results of recent investigations of FLU (4) substantiate the fact, that bacteriophages do not act in the self-purification of water (3).

All further tests for *B. coli* and for Streptococci made from November 2nd, 1938 till March 20th, 1939, from November 7th, 1939 till March 19th, 1940 and from September 24th, 1940 till March 31th, 1941 are collected in tables 1 and 2.

These tables clearly show:

1°. In prefiltered water as well as in slow sand filtered water the numbers of *B. coli* and of faecal Streptococci are increasing at low water temperature (2—3° C.).

2°. In slow sand filtered water Streptococci appear later in the year than *B. coli* and as soon as the ice in the filters is melted, they disappear at a much earlier date than *B. coli*.

Table 1.

1938-1939	PREFILTERED WATER										SLOW SAND-FILTERED WATER						
DATE	BACT COLI					STREPTOCOCCI					BACT COLI			STREPTOCOCCI			TEMP
	0,04	0,2	1	5	25	0,04	0,2	1	5	25	5	25	100	5	25	100	
NOV 2	-	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	8,9
4	-	-	+	+	+	-	-	-	+	+	-	-	+	-	-	-	10,9
9	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	10,6
21	-	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	8,8
24	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	8,1
28	-	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	6,5
30	-	-	+	+	+	-	-	-	+	+	-	-	+	-	-	+	6,6
DEC 5	-	-	+	+	+	-	+	+	+	+	-	-	+	-	-	+	6,1
7	-	-	+	+	+	-	+	+	+	+	-	+	+	-	-	+	6,3
12	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	7,0
14	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-	6,9
19	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	0,3
21	-	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	0,3
27	-	-	+	+	+	-	-	+	+	+	-	+	+	+	+	+	0,4
28	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	0,3
JAN 2	-	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,4
4	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	0,4
9	-	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	1,3
11	-	-	+	+	+	-	-	+	+	+	+	+	+	-	-	+	2,9
16	-	-	+	+	+	-	-	-	+	+	-	+	+	-	-	+	6,1
23	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	+	6,9
25	-	-	-	+	+	-	-	-	+	+	-	+	+	-	+	+	5,9
30	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	3,2
FEB 6	-	-	-	+	+	-	-	-	+	+	-	+	+	-	-	+	2,2
8	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	+	2,7
13	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	5,3
15	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	5,0
27	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	5,4
MARCH 13	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-	6,1
20	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	4,5

1939-1940	PREFILTERED WATER										SLOW SAND-FILTERED WATER						
DATE	BACT COLI					STREPTOCOCCI					BACT COLI			STREPTOCOCCI			TEMP
	0,04	0,2	1	5	25	0,04	0,2	1	5	25	5	25	100	5	25	100	
NOV 7	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	7,6
13	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	9,4
14	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	9,4
20	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	8,9
21	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	8,7
27	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	+	7,5
28	-	+	+	+	+	-	-	-	+	+	-	+	+	-	+	+	6,9
DEC. 4	-	-	-	+	+	-	-	-	+	+	-	+	+	-	-	-	7,1
5	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-	6,5
11	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-	6,2
12	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-	5,0
18	-	+	+	+	+	-	-	+	+	+	-	+	+	-	+	+	1,6
19	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	1,8
27	-	+	+	+	+	-	-	-	+	+	-	+	+	-	-	+	2,0
JAN. 2	-	-	+	+	+	-	-	-	+	+	-	+	+	-	-	+	0,9
3	-	+	+	+	+	-	-	-	+	+	-	+	+	-	-	+	1,1
8	-	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	0,6
9	-	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,5
15	-	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,7
16	-	-	+	+	+	-	-	-	+	+	+	+	+	-	+	+	0,5
22	-	-	+	+	+	-	-	-	+	+	+	+	+	-	-	+	0,4
23	-	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,4
29	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	0,4
30	-	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,3
FEB. 5	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	0,4
6	-	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,6
12	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	0,3
13	-	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,3
19	-	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+	0,4
20	-	-	-	+	+	-	-	-	+	+	-	+	+	-	+	+	0,4
26	-	-	-	+	+	-	-	+	+	+	-	+	+	-	+	+	0,7
27	-	-	-	+	+	-	-	-	+	+	-	+	+	-	+	+	1,0
MARCH 4	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	2,3
5	-	-	+	+	+	-	-	-	+	+	-	+	+	-	+	+	2,6
11	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	+	4,5
12	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	5,7
18	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	6,9
19	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	7,6

Table 2.

1940-1941	PREFILTERED WATER										SLOW SAND-FILTERED WATER						
DATE	BACT COLI					STREPTOCOCCI					BACT COLI			STREPTOC			TEMP.
	0,04	0,2	1	5	25	0,04	0,2	1	5	25	5	25	100	5	25	100	
SEPT 24	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	14,9
25	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	14,7
30	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	12,9
OCT 2	-	-	+	+	+	-	-	+	+	+	-	-	-	-	-	-	12,0
7	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	13,1
8	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	12,4
14	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	12,7
15	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	12,9
21	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	13,1
22	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	13,5
28	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	7,7
29	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	7,2
NOV 4	-	-	+	+	+	-	-	+	+	+	-	-	-	-	-	-	9,3
5	-	-	+	+	+	-	-	+	+	+	-	-	-	-	-	-	9,1
11	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	7,5
12	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	8,1
18	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	6,7
19	-	-	+	+	+	-	-	-	+	+	-	-	+	-	-	-	7,1
25	-	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	7,4
26	-	-	+	+	+	-	-	-	+	+	-	-	+	-	-	-	7,5
DEC 2	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	4,0
3	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	4,2
9	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-	4,2
10	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-	4,2
16	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-	1,5
17	-	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	2,0
20	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	2,2
23	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	0,8
30	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	0,9
31	-	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	1,4
JAN 6	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0,3
7	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	0,3
13	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	0,4
14	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	0,5
20	-	-	+	+	+	-	-	+	+	+	-	+	+	+	+	+	0,3
21	-	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	0,6
27	-	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	0,3
28	-	+	+	+	+	-	-	+	+	+	-	+	+	-	-	+	0,4
FEB 3	-	-	+	+	+	-	-	-	+	+	-	+	+	-	+	+	0,5
4	-	-	+	+	+	-	-	+	+	+	-	+	+	-	-	+	0,5
10	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	+	1,4
11	-	-	+	+	+	-	-	-	+	+	-	-	+	-	-	-	1,6
17	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-	3,7
18	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-	4,4
24	-	-	+	+	+	-	-	+	+	+	-	-	-	-	-	-	3,1
25	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-	3,1
MARCH 3	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	6,2
4	-	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	6,1
10	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	6,6
11	-	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	6,4
17	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	6,4
18	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	6,0
24	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	6,1
25	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	6,4
31	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	6,1

3°. Small quantities of slow sand filtered water, *e.g.* 5 ml, contain Streptococci only during periods of very severe frost, when the temperature of the water sinks below 1° C.

4°. Streptococci occur in prefiltered and slow sand filtered water in lesser numbers than *B. coli.*

Louwe Kooymans determined the death rate of faecal Streptococci and *B. typhi* and found that the latter die off much earlier than the Streptococci and these in their turn much earlier than *B. coli.* In the presence of protozoa (*e.g.* the Ciliate *Uronema nigricans* in a quantity of 75 per ml) typhoid bacteria (360.000 per ml) disappeared in 3 days, Streptococci (320.000 per ml) in about a week. He therefore concludes, that water, whenever free from Streptococci, will very probably be free from typhoid bacteria as well.

In the same way Stokvis and Swellengrebel (13) have recorded that *Colpoda cucullus* and *Bodo ovatus* were able to clarify turbid suspensions of typhoid bacteria completely in a few days. They stated, however, that no clarification occurred at temperatures lower than 6° C.

The above facts substantiate the conclusion, which I have drawn already in a former paper, *viz.*, that especially in winter, when *B. coli* and faecal Streptococci may appear in slow sand filtered water, indicating that the bacteriological situation is dangerous, properly applied and bacteriologically controlled chlorination of the water is essential.

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THE COMPLEMENT FIXATION TEST IN VACCINIA WITH ANTIGENS FROM THE BRAIN, THE TESTIS AND THE SKIN

by

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1. INTRODUCTION.

Since JOBLING (8) discovered that the bloodserum of vaccinated calves contains complement fixing antibodies against vaccinia virus, many investigators have looked for the occurrence of such antibodies in human blood after either vaccination or an infection with smallpox. Viewing the results of a considerable number of investigations, it may be accepted as a fact, that these antibodies are of regural occurrence although during a fairly short period and with individual variety in amount. According to PARKER (14) the antibodies appear in vaccinated rabbits 6—11 days after vaccination. The highest titre is attained after 3—6 weeks, followed by a gradual fall. The complement fixation test however may persist for 20—80 weeks. In men the antibodies cannot be detected over such a long period. In variola GASTINEL (cf. LENTZ and GINS (12)) noted them to disappear as soon as the patients were recovering. He considered a positive complement fixation test as a symptom of infection and not of immunity. In his opinion the disappearance of the complement fixing antibodies would coincide with the appearance of the virucidal ones. Later workers, however, could detect the latter antibodies also at an early date, *viz.* on the 4th day after infection. In my investigations I could detect virucidal antibodies as well before the occurrence of complement fixing ones. Both may occur simultaneously, but the complement fixing antibodies disappear more rapidly. When all experiments are considered, the results arrived at by the various workers appear to be at variance with each other. It is obvious that the length of the period elapsed since the vaccination or the onset of the disease plays an important part. Moreover the method followed in preparing the antigen influences the degree of success in the obtaining of a positive complement fixation test.

Primarily a saline extract of calf-lymph has been used (JOBLING (8), BERMBACH (1), XYLANDER (20), VAN DE KASTEELE (9)). SUGAI (17) however has used the contents of vesicles and blood from smallpox patients. KRYLOFF (10) obtained no results with organ-extracts; with saline extracts from pustules he did however. Extensive investigations are carried out by GORDON (7) with saline extracts from vaccinia of calf or rabbit. He considers the complement fixation test as strictly specific in variola, vaccinia and alastrim.

Some workers consider the reactions with an antigen derived from the skin as not reliable, as they might be the result of the association of bacterial antigens from the lymph with antibodies against the latter. According to VAN DE KASTEELE (9) however this is never the case. Nevertheless the preparation of bacteria-free antigens came into use.

KRAUS and TAKAKI, TAKAKI, BONIS and KOREF (18) prepared a koktoantigen of neurovaccine by means of which they were able to detect complement fixing antibodies in the bloodserum and in the brain of children which had died from postvaccinal encephalitis. As a rule the usual saline extract of neurovaccine does not serve, because of its anti-complementary action. Only THOMPSON, HAZEN and BUCHBINDER (19) would have obtained some results with an extract of dried rabbit-brain, in a dilution of 1 : 1200.

Servicable antigens have been prepared from culture-virus (GILMORE (6), MYERS and CHAPMAN (13), KUNERT and WENKEBACH (11)), and from a suspension of elementary bodies (FINLAYSON (4)).

Possibly the best results may be obtained with testi-vaccine-antigen, as recent investigations indicate. PARKER and MUCKENFUSS (15) used a 1% saline extract from testis of rabbits, which had been inoculated with neurovaccine and dried in vacuo, whereas MYERS and CHAPMAN (13) made a successful use of a 20% saline extract of fresh or glycerinated infected testes.

CRAIGIE (2) i.a. had detected soluble antigenic substances of specific nature (S.S.S.) in infected tissue. The more intense is the destruction the tissues are subjected to, the more S.S.S. will pass into the liquid. Therefore MYERS and CHAPMAN made an extract in a hypertonic salt solution which was diluted down to an isotonic one after 48 hours.

Similar results may be obtained by twice or thrice freezing and thawing of the suspension.

It might be important to continue the complement fixation test during a longer period than is usual in tests with bacterial antigens. In fact in virus diseases the antigen is prepared from infected tissue in which the concentration of the virus is relatively small; as a result the complement fixing ability will be lower than with bacterial antigens (cf. van Rooyen and Rhodes (16)).

The test has a twofold diagnostic value:

a. to detect antibodies in vaccinia and variola.

b. to detect antigen in suspected cases of variola.

Craigie doubts the value of *a*, because of the small amount of antibodies in the blood. The diagnostic value of *b* is more important. The S.S.S. always occurs in large amounts in the vaccinal and variolal skin lesions and this antigen may induce with hyperimmune serum a specific complement fixation test. Gildemeister and Heuer (5) could detect by means of the complement fixation test vaccinia-antigen in the brain of children which had died of post-vaccinal encephalitis.

I prepared some vaccinia-antigens from the infected brain, testes and skin of rabbits and compared their effect in the complement fixation test. The sera had been derived from:

a. rabbits used for intradermal virus titration.

b. rabbits used for intradermal neutralization tests.

c. rabbits, monkeys and men, vaccinated in the usual way.

d. rabbits hyperimmunized by intravenous vaccine injections.

The sera (diluted 1 : 5) are examined on various days after the inoculation of the vaccinia virus. The complement (guinea-pig serum) is added in 4 quantities varying from the M.H.D. or 0.5% higher to the double of this dosis. Control tests are carried out with normal sera and antigens from normal tissues. The results of the reactions are marked from 0 up to 4; (0 is complete hemolysis, 4 is complete fixation of the complement). The test has been read after an hours incubation at 37° C. and a stay overnight at room temperature.

2. ANTIGENS FROM THE BRAIN.

These have been prepared from the brain of rabbits which had been inoculated intracerebrally with neurovaccine. The brain had been removed aseptically immediately after death.

Antigen I: The brain is crushed, diluted with saline 1 : 25, 1 : 50 and 1 : 100, shaken for 5 hours and centrifuged during 15 minutes at 5000 r.p.m. The supernatant fluid serves as antigen.

A dilution lower than 1 : 50 did not serve, because of its anticomplementary action. The 1 : 50 and 1 : 100 dilutions gave a merely slight complement fixation, when hyperimmune rabbit serum was used. The control tests with normal brain and normal serum were negative.

Antigen II: The brain is crushed and dried in a vacuum exsiccator over sulphuric acid at room temperature. After 24 hours the material is extracted with ether or alcohol ether in a Sohxlet apparatus during 3 hours. Subsequently the ether is evaporated of by incubating the thimble containing the brain at 37° C. The dried powder is weighed, ground in a sterile mortar and diluted with saline to 1 : 25, 1 : 50 and 1 : 100. The latter suspensions after having been kept for some hours at room temperature are thrice frozen followed by thawing and finally centrifuged for 5 minutes at 3000 r.p.m. The supernatant fluid serves as antigen.

Following this method an antigen was prepared from the brain of various rabbits; only once however, an active antigen has been obtained. The results of the complement fixation test are registered in table 1. The sera were tested 10—30 days after the last immunization.

Table 1.
Brain antigen II.

Dilution of antigen		1 : 25			1 : 50			1 : 100			normal brain 1 : 25		
Minimal dosis complement		5%			5%			3%			5%		
% complement		5	6	8	5	6	8	3	4	5	5	6	8
Sera	Immunization												
Rabbit 8	intradermal neurovaccine	4	1	0	3	0	0	1	0	0	0	0	0
Rabbit 20	intradermal neurovaccine	4	4	1	4	3	1	3	2	0	0	0	0
Rabbit 22	hyperimmune neurovaccine	4	4	4	4	3	1	3	2	1	0	0	0
Rabbit 29	2 × vaccinated neurovaccine	4	4	1	4	3	0	3	2	0	2	1	0
Normal I		0	0	0	0	0	0	0	0	0	0	0	0
Normal II		2	0	0	2	0	0	0	0	0	0	0	0

The reactions are rather slight, the more so in the higher dilutions of the antigen. The control tests are negative. The sera from 4 men, who had been vaccinated 10 days earlier, gave negative results as well.

3. ANTIGENS FROM THE TESTIS.

Antigen III: Both testis of a rabbit are removed on the fourth day after an intratesticular inoculation with neurovaccine. The material is crushed and diluted with saline to 1 : 25, 1 : 50 and 1 : 100. After having been kept at room temperature for some hours, the suspensions are centrifuged for half an hour at 2500 r.p.m. The supernatant fluid serves as an antigen.

The complement fixation test has been carried out with 26 immunesera of rabbits, serum of a vaccinated monkey and 12 sera of normal rabbits. The sera were tested in the period of 6 to 108 days following the vaccination. The reaction with the immunesera was from the 6th to the 14th day invariably marked. Later on it varied with the individual, but even on the 108th day some animals among which the monkey still had a positive reaction. It is not needed to use a dilution of the antigen under 1 : 100, as the reaction in this case is distinct as well as specific. All control tests were negative.

The reaction has also been carried out with sera of some of the same animals, their immunity having been raised by intravenous vaccine injections. This treatment increased the amount of complement fixing antibodies somewhat. The highest concentration was reached after 3 injections. More injections had no effect (table 2).

Table 2.
Testis antigen III (1 : 100).

	first immunization				1st intravenous injection				2nd intravenous injection				3rd intravenous injection			
Minimal dosis complement	5%				5%				5%				5%			
% complement	5½	7½	9	10	5½	7½	9	10	5½	7½	9	10	5½	7½	9	10
Sera																
Rabbit 216	4	4	4	2	4	4	4	3	4	4	4	4	4	4	4	4
Rabbit 222	4	4	4	2	4	4	4	2	4	4	4	2	4	4	4	3
Rabbit 232	4	4	2	0	4	4	4	2	4	4	4	2	4	4	4	3
Rabbit 234	4	3	0	0	4	3	1	0	4	3	3	2	4	4	3	0
Normal 1	0	0	0	0												
Normal II	0	0	0	0												
Normal III	0	0	0	0												

Antigen IV: The testis of a rabbit are removed on the 4th day after an intratesticular inoculation of neurovaccine, crushed and dried in a vacuum exsiccator over sulphuric and phosphoric acid. After 24 hours the material is weighed and transferred to a flask containing 300 ml ether, closed with a rubber stopper. After having been shaken twice or thrice for half an hour, the ether is poured off. The tissue is kept at 37° C. in order to let the rest of the ether evaporate. The dried powder is diluted then with 0.9% saline to 1 : 25, 1 : 50 and 1 : 100. The suspensions are kept at room temperature overnight and are thrice frozen and thawed down on the following day. After having been centrifuged for 5 minutes at 4000 r.p.m. the supernatant fluid serves as antigen.

In this method CRAIGIE and TULLOCH (3) are followed in their method of preparing an antigen from the skin. The effect of this antigen is inferior to that of the former (table 3).

Table 3.

Antigen		III				IV				V				normal testis			
Minimal dosis complement		5%				7%				5%				5%			
% complement		5½	7½	9	10	7½	10	12	14	5½	7½	9	10	5½	7½	9	10
Sera	Immunization																
Rabbit 153	intradermal 0.1 ml (1 : 100)	4	4	2	1	4	2	2	0	4	4	4	2	0	0	0	0
Rabbit 222	virus titration	4	4	2	2	4	2	1	0	4	4	4	4	0	0	0	0
Rabbit 216	intravenous 0.25 ml (1 : 50)	4	4	2	2	4	4	2	0	4	4	4	4	0	0	0	0
Rabbit 234	neutralization test	4	3	0	0	4	0	0	0	4	4	4	3	0	0	0	0
Rabbit 259	vaccinated	4	3	2	0	4	2	0	0	4	4	4	1	0	0	0	0
Monkey 19	vaccinated	4	4	4	2	4	4	2	2	4	4	4	3	0	0	0	0
Normal I		0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Normal II		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Normal III		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The best results may probably be obtained with antigen V, which is prepared by a partial combination of the methods for antigens III and IV, with the omission of the ether extraction.

Antigen V: The inoculated testis are crushed with sterile sand, diluted down to 1 : 100. After remaining overnight at room temperature the material is thrice frozen and thawed again and centrifuged for 15 minutes at 2500 r.p.m. This antigen may serve during 2—4 weeks, provided it is stored in the ice box.

Table 3 shows the results of the complement fixation tests with the antigens III, IV and V and with a normal antigen prepared like V.

4. ANTIGENS FROM THE SKIN.

The reactions with three of these antigens are less marked than those with the antigens from testis (table 4).

Antigen VI: Calf lymph is crushed with sterile sand and diluted with 0.9% saline to 1 : 100. After having been kept at room temperature for some hours, the suspension is centrifuged for 5 minutes at 2000 r.p.m. The supernatant fluid serves as the antigen.

Antigen VII and VIII: Vaccinia of a rabbit having been vaccinated with neurovaccine, respectively dermovaccine is dried,. treated with ether and diluted to 1 : 100 such as it has been described for antigen IV.

Table 4.
Antigens from the skin (1 : 100).

Antigen	VI				VII				VIII			
Minimal dosis complement	4%				3%				3%			
% complement	4	5	6	8	3	4	5	6	3	4	5	6
Sera												
Rabbit 216	4	3	1	0	4	2	1	0	4	2	2	0
Rabbit 222	4	4	4	2	4	2	0	0	3	1	0	0
Rabbit 232	1	0	0	0	2	1	0	0	2	0	0	0
Rabbit 234	3	2	0	0	2	0	0	0	2	1	1	0
Normal I	0	0	0	0	0	0	0	0	0	0	0	0

When the action of the various antigens is compared, it can be noted, that the best results are induced by the 1% saline extract of fresh testis. The sterile preparation is an easy matter. The reactions have been carried out with antigens derived from various rabbits; no individual influence however can be noted. The amount produced by a single rabbit is high and may attain to almost 400 ml. When frozen this antigen may be kept for some weeks.

The complement fixing antibodies may be detected in rabbits as early as on the 6th day following vaccination. The highest titer is reached on the 12th—14th day; then it decreases rather rapidly. Individual differences however occur.

Usually the reaction is still positive after three months, although considerably decreased. The titer of the serum is more or less dependent on the degree of vaccination. An extensive vaccination will generally give rise to a higher amount of antibodies than a limited one. 20 sera of revaccinated men have been examined with antigen V. The revaccination had taken place from three months to many years earlier. It is not to be wondered at that the reactions were not very marked. Some however were more or less positive, *viz.* one with the serum of a person who had been revaccinated 8 years earlier. In variola perhaps the reaction may only be of diagnostic value during, or immediately after the disease.

Summary.

Eight antigens, prepared from the brain, the testis and the skin of rabbits after the tissues had been inoculated with vaccinia virus, have been compared in the complement fixation test with vaccinia-immune-sera. It was rare that a serviceable antigen could be prepared from the brain. Antigens from the testis and the skin were always active, but the activity of the former surpassed that of the latter. The best results were obtained with a saline extract 1 : 100 of freshly removed testis; when frozen this extract may be kept for some weeks. In the sera of vaccinated rabbits and of a monkey complement fixing antibodies could be detected regularly, even 3 months after vaccination. In sera of men, who had been revaccinated more than 3 months earlier, this was only rarely the case.

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(From the Microbiological Laboratory, Agricultural University, Wageningen).

BACILLUS AGAR-EXEDENS, A NEW SPECIES, DECOMPOSING AGAR

by

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The first to recognise the assimilation of agar by bacteria was GRAN (3), who in 1902 gave a description of *B. gelaticus*, which had been isolated from seaweeds off the Norwegian and Dutch coasts. Some years later PANEK (6) isolated from beets *B. betae viscosum*, which could hydrolyse agar, and in 1910 *B. Nenckii* was isolated by BIERNACKI (1) from Malaga raisins. The látter organism can form gas and acids from various carbohydrates. In 1924 GRAY and CHALMERS (2) found a micro-organism which could decompose cellulose as well as agar. It was called *Microspira agar-liquefaciens* (= *Vibrio agar-liquefaciens* (Gray and Chalmers) Bergey et al. See also VAN DER LEK (5)). All of these papers dealt with non-sporulating bacteria.

In an early attempt to isolate *V. agar-liquefaciens* from soil a student in this laboratory found a bacterium hydrolysing the agar. To our surprise however production of spores could be stated microscopically. A pure culture (strain 8.61), which was obtained after a series of subcultures showed, that spore production was a feature of this microbe and that our observation could not be ascribed to a contaminating organism.

The symptoms of the hydrolysis of the agar by this organism are unlike those by *V. agar-liquefaciens*, which liquifies the agar; they resemble however those described by GRAN. In a zône surrounding the colonies the agar is more transparent than in the further plate. No liquefaction in a strict sense is observed. The agar is merely softened in the transparent zône in the centre of which the colony is lying in a hollow with gentle slopes (cf. fig. 1). It is for this reason, that the name *agar-liquefaciens* cannot be applied to this micro-organism. I will call it subsequently *Bacillus agar-exedens* (from *exedo* = I hollow out by eating). In the transparent zône the agar does not react with iodine, whereas the undecomposed agar stains violet with this reagent. Bacteria treated with chloroform induce the same symptoms when brought in lumps upon agar plates.

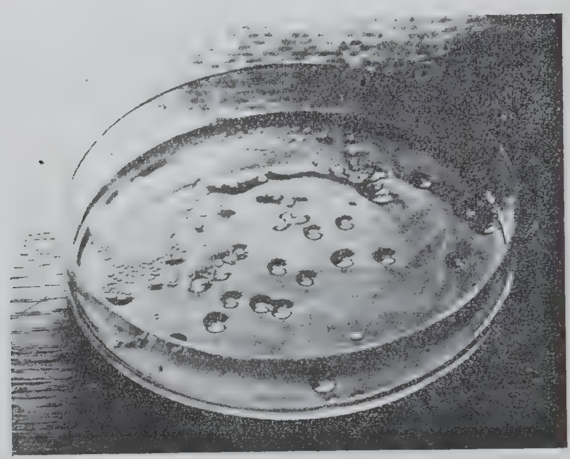

Fig. 1.
Colonies of *Bacillus agar-exedens* on tap-water agar.

A number of writers have tried to ascertain the reaction products of the decomposition of the agar. GRAN thinks that agar is a mixture of two components, one of which reacts with iodine to form a violet compound, whereas the other remains colourless. The former substituent, after desintegration by the bacteria is formed overnew by heating the latter. Several writers have stated the formation of substances reducing the Fehling solution. I however could observe reduction of the Fehling solution to a merely very slight degree when cultures on agar plates were heated with the reagent at a temperature near 70°C. A brown precipitate was formed in the agar outside of the transparent zône.

The auxanographic method using *B. phosphorescens* as an indicator organism has been successfully applied by GRAN in order to show the formation of sugar. I tried the same method using *Saccharomyces cerevisiae*, but without any success. Perhaps a sugar is formed that cannot be assimilated by *S. cerevisiae*. Yeast extract agar was used as a culture medium in this experiment.

In agar plates coloured with litmus the formation of acid from agar can be easily demonstrated. I have not yet tried to determine the nature of this acid. As agar appears to be an ester of sulfuric acid, this acid may possibly be set free by hydrolysis. I could not, however, observe the merest indication of a precipitate when acidified $BaCl_2$ was poured upon an agar culture. Even when the concentration of the agar had been brought up to 5% not the slightest amount of precipitate could be seen in 10 days old cultures. Most probably the acid originates from the break-down of the carbohydrates and allied substances which build up the agar molecule. These are essentially galactose, laevulinic acid and uronic anhydride. *Bac. agar-exedens* can form acid from glucose, galactose, saccharose, maltose and lactose.

Two other strains of *Bac. agar-exedens* have been isolated subsequently from leafmould (8.63) and from dung (8.64). Suspensions of these substances were inoculated in solutions containing 0.1% agar, 0.1% NH_4Cl and 0.1% K_2HPO_4 in tap-water. After having been kept 2 or 3 days at 30°C. these cultures were heated for 5 minutes at 80°C. and sown on tap-water agar plates with the above mineral additions. A fourth strain (8.62) had already been isolated incidentally from a Liebig broth agar plate, which had intentionally been exposed to air-infection in a students course. All of these strains showed to possess the above cultural characteristics. In one point however there is a marked difference. The strains from soil, leafmould and dung will not grow on the bouillon-peptone agar. This point will be treated in detail further down.

Starch is hydrolysed readily by either strain. Cellulose however is not hydrolysed. A suitable medium for demonstration of the decomposition of carbohydrates other than agar is the silicogel plate, made up using a technic similar to that of HETTCHE and MÜNCH (4). To 1000 ml of distilled water, 1 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4$, traces of $FeCl_3$ and $MnSO_4$ and 10 to 20 g of the carbohydrate are added. This solution is sterilised (Sol. I.). To 200 ml of solution I 2 ml of bromo-thymol blue and 12 ml of water glass are added and the pH is brought to 7 by addition of 25% H_3PO_4 (II.). A stock solution (III) is now prepared consisting of 1000 ml of Sol. I and 5 × the amount of H_3PO_4 determined in II. A mixture of 100 ml of III with 6 ml of water glass will solidify within half an hour. Not the slightest dissimilation of cellulose could be observed in this medium in a fortnight.

Other cultural characters inherent to either strain are the following: acid but no gas is formed from sugars, gelatin is not liquefied, litmus milk is coloured brick red in two days, no curd is formed in milk, casein is not peptonised, nitrite is not formed from nitrate, no indole is formed in peptone broth. All strains are obligately aerobic. Stab cultures in agar will only grow upon the surface and perhaps 1 or 2 mm in the stab.

There is one marked difference between strain 8.62 (air infection) and the strains 8.61, 8.63 and 8.64 (isolations from soil, leafmould and dung respectively). The first does grow readily on peptone or bouillon broth, whereas the latter 3 either do not grow on these substrates or, if so, the growth is very greatly retarded. In 3 weeks a small number of colonies may develop on Liebig bouillon agar plates, when these have been sown with a heavy suspension of bacteria.

Other nitrogen compounds were tried as nutrition for these bacteria. Among these NH_4^+, nitrate, urea and asparagine gave a good growth in a sugar medium. With uric acid no growth was obtained. The inorganic nitrogen compounds however are but poor N-sources for strain 8.62. In a solution of these compounds in tap-water with 1% glucose, 0.1% K_2HPO_4, 0.1% $MgSO_4$ and a trace of $FeCl_3$ this strain does not grow. In soil extract with these additions the growth is limited to a slight turbidity.

A further experiment showed that presence of peptone (peptone Witte was used in these experiments) impedes the growth of the strains 8.61, 8.63 and 8.64 even when appropriate N-sources are present, the only exception being urea. To a solution containing 1% glucose, 0.1% K_2HPO_4 and 0.5% peptone, 0.1% of various N-sources were added. The effect of the presence of peptone follows from table 1.

Table 1.

Effect of the presence of peptone on 4 strains of *Bac. agar-exedens.*

N-source	No. of strain			
	8.61	8.62	8.63	8.64
NH_4Cl	—	++	—	—
KNO_3	—	++	—	—
Asparagine	—	++	—	—
Urea	+	++	+	+
Uric acid	—	++	—	—
—	—	++	—	—

It is remarkable that growth is possible with peptone when urea is present. Smaller amounts of peptone are already injurious as is shown in table 2. Two stock solutions, one containing 1% glucose, 0.1% K_2HPO_4, 0.1% NH_4Cl and 0.05% $MgSO_4$ in tap-water, the other the same substances and 1% peptone, were mixed in various proportions. The solutions were inoculated with the strains 8.61 and 8.62.

Table 2.

Effect of the presence of peptone on two strains of *Bac. agar-exedens.*

% Peptone	No. of strain	
	8.61	8.62
0	+	+
0.2	—	+
0.4	—	+
0.6	—	+
0.8	—	+
1.0	—	+

Morphologically *Bac. agar-exedens* (fig. 2 and 3) is a rod of variable length, with rounded ends, motile by means of peritrichous flagella, mostly single, sometimes pairs or short chains may be observed. Of each strain 40 cells, stained with Loeffler's methylene blue were measured. The result is given in table 3.

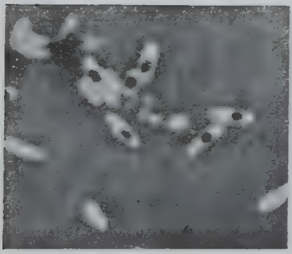

Fig. 2.
Spores in *Bacillus agar-exedens* stained by DORNERS method (1000 ×).

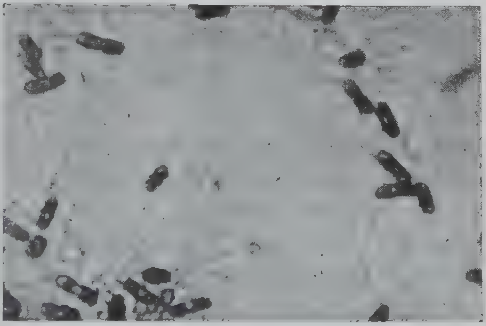

Fig. 3.
Flagella of *Bacillus agar-exedens* stained by PEPPLER's method (1000 ×)

Table 3.

Length of the cells of different strains of *Bac. agar-exedens.*

No. of strain	Length of bacteria	
	Varying between	Middle
8.61	3—20 μ	10.9 μ
8.62	3—8 μ	5.3 μ
8.63	3—17 μ	6.5 μ
8.64	3—10 μ	7.0 μ

Oval spores are found mostly at the end of the bacilli. Clostridial forms are common at sporulation. Very often the bacilli are slightly curved. *Bac. agar-exedens* stains positively with Gram's staining method.

In order to check my observations so far made, Mr. D. S. TUINMAN, a student in this laboratory was asked to isolate agar liquefying bacteria from a number of different soils. Among these were sandy and peaty soils with different manuring, a heavy loam and leaf mold. Agar liquefying bacteria could be isolated especially from soils containing much organic substances and from leafmold. In no case Mr. TUINMAN was able to isolate non-sporulating forms, such as *V. agar-liquefaciens.* In most of his raw cultures bacteria were common that were microscopically identical with *Bac. agar-exedens.* A strain isolated in pure culture was very motile in young cultures, rods measuring 3—8 μ in length and 1.5 μ in width are often swollen Clostridia-like at sporulation. The flagella are peritrichous. Physiologically this strain was quite identic with the strains 8.61, 8.63 and 8.64. It does not grow with peptone. Two other strains however were isolated of a somewhat different character. One of these was different only in size, being 2—6 μ long and 1 μ wide. Physiologically this strain is identical with the former; it does not form acid from agar however.

The third strain isolated by Mr. TUINMAN is much smaller (1—2.5 μ long, 0.8 μ wide), non motile with rounded ends. The spores are terminal and round, whereas all other strains have oval or cylindrical spores. It does grow very well in peptone solutions. In old cultures curved forms are common, vibrio-like, but non motile.

None of these strains liquefies the agar in a strict sense.

Conclusions.

In stable manure, leafmold and soils (especially those with a high content of organic material) agar decomposing bacilli are relatively common. Different types of these organisms were isolated, the type described as *Bacillus agar-exedens* being the one most frequently met. The occurrence of these bacteria in materials with a high content of organic matter suggests the presence in these materials of higher organic compounds, especially apt for their nutrition.

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(Aus der Abteilung Infektionskrankheiten des Instituts für Parasitäre- und Infektionskrankheiten der Reichsuniversität zu Utrecht).

CLOSTRIDIUM PERFRINGENS INFEKTIONEN DES VERDAUUNGS- UND UROGENITALAPPARATES

von

JAC. JANSEN

(Eingegangen am 11 Juli 1941).

Einleitung.

Clostridium perfringens (*Clostridium welchii*) ist der sehr bekannte anaërobe sporenbildende Bazillus, der überall im Erdboden vorhanden ist. Dieser Mikroorganismus gehört mit noch einigen *Clostridium*-Arten wie z.B. dem Rauschbrandbazillus zu der Gruppe der sog. Gasödemerreger. Es ist bekannt, dass *Cl. perfringens* (in der deutschen Literatur: „FRAENKELscher Gasbazillus") für verschiedene Tierarten und besonders auch für den Menschen hoch pathogen sein kann. Über die Pathogenität für den Hund ist wenig oder nichts bekannt. Im Juni 1940 und danach im Februar 1941 wurde dieser Bazillus von mir beim Hunde gefunden, und in beiden fällen führten die Umstände zu der Überzeugung, dass *Cl. perfringens* eine pathogene Bedeutung besessen hatte, sodass eine Erwähnung dieser Fälle vielleicht von Nutzen sein kann, zumal man in der Literatur darüber praktisch nichts findet.

In dem bekannten Handbuch von VON HUTYRA, MAREK und MANNINGER (1) wurden Infektionen mit *Cl. perfringens* beim Hunde nicht erwähnt, und ebenso wenig findet man etwas in KELSER's „Manual of Veterinary Bacteriology" (2). Auch in „The Principles of Bacteriology and Immunity" von TOPLEY und WILSON (3), in „Traité des Maladies Infectieuses et Contagieuses" von FORGEOT (4), in ZEISSLERS „Anaerobenzüchtung" (5), in BERGEY's „Manual of Determinative Bacteriology" (6) und in „A system of Bacteriology" von ROBERTSON (7) wurden keine Infektionen von *Cl. perfringens* beim Hunde genannt. In „A textbook of general Bacteriology" von JORDAN (8) werden ziemlich viele Tierarten, für welche die Empfänglichkeit für *Cl. perfringens* fest steht, nicht genannt, aber gerade der Hund wohl; diese Tatsache und die Mitteilung, dass „local abscesses have been observed in dogs and rabbits following injury" (*Cl. perfringens* verursacht diffuse Gasödemphlegmone) lässt diese Angabe zweifelhaft erscheinen, die vielleicht zurückzuführen ist auf das eingehendste Werk auf dem Gebiete der Anaëroben-Bakteriologie, nämlich das von WEINBERG, NATIVELLE und PRÉVOT: „Les Microbes Anaérobies" (9); über den Hund wurde darin allein gesagt: „Chien. — Abcès du cou sans infiltration gazeuze (HARRIS)". Die Arbeit von HARRIS wird aber nicht genannt, und ebenfalls konnte nicht festgestellt werden, ob es sich bei diesem HARRIS um denselben Autor handelt, der in dem Vorwort von JORDANS Buch als Mitarbeiter genannt wird. Das französische Buch sagt ausdrücklich: „sans infiltration gazeuze", sodass in diesem Fall also keine typische Infektion mit *Cl. perfringens* mit „infiltration gazeuse" beobachtet wurde; die Möglichkeit besteht (*Cl. perfringens* ist der am meisten ubiquitäre Bazillus und ist auf der Haut stets vorhanden), dass ein oberflächlicher Halsabszess von *Cl. perfringens* infiziert wurde. TEUNISSEN (10) wies im Uterusinhalt eines Hundes *Cl. perfringens* zusammen mit Streptokokken und Colibazillen nach; der Entzündungsprozess des Uterus war subakut und gutartig, auch hier war also keine Rede von einem durch *Cl. perfringens* erzeugten Gasphlegmon. Das Vorhandensein dieses Mikroorganismus das wegen der offenen Verbindung zwischen dem Uterus und der Aussenwelt, in der *Cl. perfringens* stets anwesend ist, ist leicht zu erklären.

Die zwei Fälle, in denen ich *Cl. perfringens* nachweisen konnte, wiesen beide gleichzeitig Abweichungen auf, die mit denen von Gasödem übereinstimmten.

1. Fall.

Anamnese: bei einem 13 Jahre alten, gestorbenen Foxterrier, bei dem ein Tumor mammae entfernt und postmortal u.a. Pyometra und geringe Nephritis festgestellt war, wurde bei der Sektion (ausgeführt von Herrn Kollegen J. H. TEN THIJE, Pathologisches Institut, Direktor Prof. Dr. H. SCHORNAGEL) ausserdem noch gefunden: „akute Gastritis, der Magen leer, die Schleimhäute geschwollen und gefleckt, grünbraun gefärbt. Exsudat wurde auf der Mukosa nicht gefunden, wohl waren überall in der Mukosa Gasblasen vorhanden. Verwesung ist ausgeschlossen, da das Tier lebenswarm seziert wurde". Da eine Infektion durch anaërobe Bazillen vermutet war, wurde der Magen der Abteilung Infektionskrankheiten für eine bakteriologische Untersuchung überwiesen.

Bakteriologische Untersuchung des Magens.

Von der geschwollenen Magenwand, in der makroskopisch die Gasentwicklung zu sehen war, wurden zunächst Ausstrichpräparate angefertigt. Hierin wurden plumpe, Gram-positive Stäbchen gesehen. Aus der Magenwand wurde, da bereits die Überzeugung erhalten war, dass es sich um eine Anaërobeninfektion handelte, auf Leberbouillon geimpft. Hierin entstand schnelles Wachstum mit reichlicher Gasentwicklung. Die Serumagar- und Serumbouillonröhrchen, die aus den positiven Leberbouillonröhrchen beimpft wurden, blieben steril, sodass allein Anaëroben gezüchtet waren. Die Leberbouillonkultur wurde übergeimpft auf eine Reihe Traubenzucker-Blutagarplatten und anaërob bebrütet. Auf diesen Platten wuchsen ausschliesslich die typischen Kolonien von *Cl. perfringens.*

Bereits nach 24 Stunden hatten sich Kolonien ohne Ausläufer, mit einer breiten hämolytischen Zone entwickelt und die anfänglich graue Farbe veränderte sich nach Blosstellung an der Luft in mattgrün. Die Kolonien bestanden aus schweren Bazillen, die morphologisch völlig mit *Cl. perfringens* übereinstimmten. In einem Dunkelfeldpräparat der Leberbouillon wurde keine Eigenbewegung beobachtet (*Cl. perfringens* ist das einzige unbewegliche pathogene *Clostridium*).

Pathogenität.

Es ist bekannt, dass die Pathogenität verschiedener Stämme von *Cl. perfringens* sehr unterschiedlich sein kann. Eine Untersuchung nach der Pathogenität dieser Kultur für Meerschweinchen hatte folgendes Ergebnis. Das intramuskulär mit Magensuspension eingespritzte Meerschweinchen und das mit Leberbouillonkultur intramuskulär injizierte Tier erhielten beide eine Reaktion, die aber später wieder verschwand. Das subkutan am Nabel mit Kulturmaterial injizierte Meerschweinchen erhielt die typischen *Cl. perfringens*-Veränderungen, aber auch dieses Meerschweinchen genas; ein grosser Teil der Nabelumgebung wurde nekrotisch und abgestossen und der entstandene Ulcus heilte darauf ab. Hierauf wurde nochmals ein Meerschweinchen am Nabel infiziert; dieses Tier wurde 24 Std. später getötet. Die Bauchwand war stark geschwollen und die plumpen unbeweglichen *Cl. perfringens*-Bazillen wurden nachgewiesen. Hiermit wurde die Untersuchung als beendet betrachtet; die Kultur wurde nicht angehalten.

2. Fall.

Anamnese: Von der Klinik für kleine Haustiere (Direktor Prof. Dr. A. KLARENBEEK) wurde von einem Hund Urin zur Untersuchung eingeschickt, da dieser hämorrhagisch war und plumpe Stäbchen enthielt.

Hierüber wurde berichtet: „Ein deutscher Schäferhund litt infolge Prostatahypertrophie an Retentio urinae. Nach Kastrierung blieben die Beschwerden noch 5 Tage bestehen. Die Blase wurde täglich mit einem Katheter geleert, der Urin war bakterienfrei. Hierauf beginnt der Hund plötzlich spontan zu urinieren. Der Urin ist hämorrhagisch und in einem Methylenblaupräparat werden viele Bakterien, kurze plumpe Stäbchen, gesehen. Die Temperatur, die erst normal war, steigt bis zu 40° C."

Bakteriologische Untersuchung des Urins.

Im Dunkelfeldmikroskop wurden im Urin sehr viele ausschliesslich grosse, plumpe, unbewegliche Bazillen gesehen, die an *Cl. perfringens* erinnerten. Ein schwer beïmpftes Röhrchen Bouillon und 5 Blutagarplatten zeigten nach einem Tage aërober Bebrütung kein Wachstum. Diese Nährböden wurden darauf anaërob bebrütet und gleichzeitig noch 5 Traubenzucker-Blutagarplatten. Nach 24 Std. war die Bouillon trübe (Dunkelfeldpräparat: ausschliesslich *Cl. perfringens* ähnliche, unbewegliche Bazillen); auf den Blutagarplatten waren nur runde Kolonien eines einzigen Typs entstanden, auf Traubenzucker-Blutagarplatten waren ausschliesslich die typischen Kolonien von *Cl. perfringens* gewachsen, nämlich runde mit breiten hell kaffeefarbenen hämolytischen Zonen; nachdem sie einige Zeit der Luft ausgesetzt waren, wurden die Kolonien mattgrün von Farbe. Die gezüchteten Bakterien waren unbeweglich, Gram-positiv und stimmten in Form mit *Cl. perfringens* überein.

Die Diagnose *Cl. perfringens*-Infektion wurde auf Grund dieser Beobachtungen gestellt. Serumtherapie wurde aber nicht mehr versucht, da der Zustand des Hundes schon derartig war, dass beschlossen wurde, das Tier zu töten. Aus dem Sektionsbericht (von Herrn Kollegen J. H. TEN THIJE, Pathologisches Institut, Direktor Prof. Dr. H. SCHORNAGEL) ergab sich, dass „die Prostata mässig vergrössert war; vorne in der rechten Hälfte wurde ein reichlich marmelgrosse, mit gelbem Eiter gefüllte Höhle gefunden. Die Oberflächenbekleidung und die Serosa in der Umgebung der Prostata wie auch das Bindegewebe im Becken war feucht und rot, als ob sich eine phlegmonöse Entzündung entwickelt hatte. In der Blasenschleimhaut waren eine Anzahl kleiner Blutungen mit dem Bilde einer Urocystitis".

Für die bakteriologische Untersuchung wurden nur Leber, Milz und Blase zugesandt. Allein aus der Blase wurde *Cl. perfringens* isoliert; die anderen Organe waren steril. Zur Sicherheit wurden noch einige Nährböden beimpft, die für die Bestimmung von Gasödembazillen von Bedeutung sind, nämlich Milch, die sehr schnell koagulierte (bereits nach einer Nacht; dies und die grosse Gasbildung, die beobachtet wurde, ist für *Cl. perfringens* typisch), Gelatine, die verflüssigt wurde, und Gehirnbrei, in dem keine schwarze Verfärbung auftrat.

Aus der Literatur ergibt sich, dass verschiedene *Cl. perfringens*-Stämme vorkommen; nach ROBERTSON (7) ist ,,*B. welchii* divided into four groups (SIMONDS, 1915) on the fermentation of inulin and glycerin". BERGEY (6) schreibt: ,,action on inulin and glycerol variable"; TOPLEY and WILSON (3) erwähnen: ,,some strains ferment inulin, some glycerol"; diese Autoren sagen über die Stammesunterschiede weiter noch bei der Besprechung der Antigenstruktur: ,,By agglutination no clear-cut grouping is apparent; considerable overlapping of antigens. No obvious relation between agglutination results and typing of strains by toxin-antitoxin or biochemical methods. Apparently four different types of exotoxin produced, sometimes all four being produced by one strain". Die Einteilung auf Grund der biochemischen Eigenschaften, die auf der Vergärung von Inulin und Glycerol beruht, unterscheidet nach KELSER (2) und FORGEOT (4) 4 verschiedene Typen, nämlich:

I. Inulin und Glycerol, beide vergoren
II. Inulin nicht, Glycerol wohl vergoren
III. Inulin wohl, Glycerol nicht vergoren
IV. Weder Inulin noch Glycerol vergoren.

Die Kultur von *Cl. perfringens* des 2. Falles gehörte dem Typ II an.

Pathogenität.

Die Untersuchung nach der Pathogenität beim Meerschweinchen verlief völlig gleichartig mit dem ersten Fall. Ein Meerschweinchen, das am Nabel subkutan mit Urin eingespritzt wurde, erhielt die typische fluktuierende *Cl. perfringens*-Schwellung und das intramuskulär eingespritzte Meerschweinchen einen enorm geschwollenen Fuss; beide Tiere blieben aber aktiv und genasen. Während dieser Heilung wurde ein grosser Teil abgestorbenen Gewebes abgestossen. Hierauf wurde ein Meerschweinchen subkutan am Nabel mit Leberbouillonkultur infiziert; auch dieses Tier erhielt lokal eine enorme, mit Feuchtigkeit und Gas gefüllte Schwellung am Bauch; das mit Leberbouillonkultur in der Schenkelmuskulatur injizierte Meerschweinchen entwickelte eine ausgedehnte, lokale Schwellung. Auch diese Meerschweinchen blieben normal aktiv und genasen. Stämme von *Cl. perfringens* können Meerschweinchen sehr schnell töten. Aus den Versuchen erhält man daher den Eindruck, dass die aus dem Hund isolierten Kulturen nicht eine optimale Pathogenität (wenigstens nicht für Meerschweinchen) besassen. Vom 2. Fall wurde mit Urin und Kultur subkutan und intramuskulär ein junger Versuchshund infiziert, aber es wurde nicht einmal eine lokale Reaktion beobachtet. Dies Fehlen einer Reaktion bei dem Versuchshund braucht aber nicht zu verwundern, da wir ein völlig gesundes Versuchstier infizierten und die infizierten Keime daher nicht durch Prädispositionen geholfen wurden, sodass das Angehen der Infektion ausbleiben konnte. Derartige negativ verlaufene Versuche, auch bei Tieren, die spontan empfänglich sein können, werden jedem, der Tierexperimente ausführt, bekannt sein (z.B. bei der experimentellen Infektion von Schweinen mit Rotlauf). Ein idealer Versuchshund wäre hier ein schwaches Tier gewesen, das an Retentio urinae litt, eine Infektion (in die Blase) wäre hierbei vielleicht gelungen.

Beurteilung.

Die obenstehenden Ausführungen zeigen, dass zweimal *Cl. perfringens* von nicht grosser Pathogenität aus einer Tierart isoliert wurde, die im Allgemeinen gerade wenig für diesen Mikroorganismus empfänglich ist (wenn dies nicht zuträfe, müssten nämlich derartige Infektionen besser bekannt sein). Ohne Zweifel muss an den Einfluss von wichtigen prädisponierenden Faktoren gedacht werden; im 1. Fall war der Hund bereits 13 Jahre alt und litt an Tumoren, Pyometra, chronischer Nephritis usw., im 2. Fall hatte der 6 bis 7 Jahre alte Hund an Prostatahypertrophie gelitten mit einer sekundären andauernden Harnverhaltung und als Folgeerscheinung eine stets zu grosse Blase; ausserdem was das Tier kürzlich kastriert und kachektisch. Eine gewisse Übereinstimmung des Falles der Blaseninfektion beim Hund besteht mit dem früher von mir beschriebenen Fall von *Cl. perfringens*-Infektion bei einem Rind, das an Hydrallantois litt (11); bei diesem Tier war nach der Entleerung des Fruchtwassers infolge der Perforation der Haut mit einem Katheter eine phlegmonartige Entzündung der Fruchthäute und des Uterus entstanden; das mit der isolierten *Cl. perfringens*-Kultur intramuskulär im Schenkel eingespritzte Versuchskalb blieb völlig reaktionslos. Bei Rindern kommen *Cl. perfringens*-Infektionen nie vor, aber in diesem Fall, in dem der Bazillus gezüchtet wurde, war ein abnormaler Zustand vorhanden; ebenso wie die Blasenwand des Hundes hatten hier der Uterus und die Fruchthäute längere Zeit unter einer unphysiologischen Spannung gestanden, während infolge des abnormalen Zustandes auch die allgemeine Resistenz des Tieres vermindert gewesen sein wird. Hier wäre an stelle eines kerngesunden Kalbes ein Rind mit Hydrallantois das ideale Versuchstier gewesen.

Über die Lokalisation des andern Falles (Magen) kann folgendes gesagt werden. Man neigt wohl dazu, um bei der *Clostridium*-Gruppe von ,,*Wundinfektion*''-Anaëroben zu sprechen und denkt hierbei an äusserliche Verwundungen; tatsächlich sind Bazillen wie *Cl. perfringens*, der Rauschbrandbazillus, *Cl. novyi* usw. gefürchtete Verunreiniger von Wunden und können schnell verlaufende, tötliche Infektionen hervorrufen, aber man vergesse nicht, dass sie, ausser im Bindegewebe und Muskelgewebe bei Wunden, hauptsächlich auch über den Verdauungsapparat den Körper infizieren können.

Was die Magenwand betrifft, sei hier daran erinnert, dass in diesem Organ Infektionen durch die Gattung *Clostridium* bekannt sind. ,,Bradsot'' oder ,,Braxy'' z.B. ist eine typische Schafkrankheit, die durch verschiedene *Clostridium*-Arten, u.a. *Cl. perfringens* hervorgerufen wird, wobei gerade kennzeichnend die akute Labmagenerkrankung ist. Bei Schweinen kommt Gastritis durch *Cl. oedematis maligni* (Pararauschbrandbazillus) (Köves) vor und, wie von mir beschrieben (12) auch durch *Cl. novyi*. Besonders bei Schafen ist der Verdauungstrakt für *Cl. perfringens* empfindlich; man kennt bei dieser Tierart ausser ,,Bradsot'' die ,,Lamb dysentery'', eine Enteritis bei Lämmern, die von *Cl. perfringens* des Toxintypes B verursacht wird; bei erwachsenen Schafen in England kennt man eine ,,Enterotoxaemie'' durch *Cl. perfringens* des Toxintypes C, genannt ,,struck'' und in Australien eine derartige Schafkrankheit, die von *Cl. perfringens* des Toxintypes D verursacht wird. Wir sehen also, dass der Verdauungstrakt keineswegs eine nur ausnahmsweise vorkommende Lokalisation für Anaëroben-Infektionen im allgemeinen und für *Cl. perfringens* im besondern ist. Denken wir nun weiter an den Fall bei dem Rinde mit Hydrallantois und den hier beschriebenen 2. Fall des Hundes (Urin), dann ist es als wahrscheinlich anzusehen, dass alle mit Schleimhäuten bekleideten Organe Infektionen mit *Clostridium*-Arten ausgesetzt sein können. Derartige Anaëroben-Infektionen des Verdauungstrakts und Urogenitalapparates dürften beim Hund wohl sporadisch vorkommen. Bei

Schafen liegen die Verhältnisse wahrscheinlich anders. Herr Kollege A. M. Hibma dachte bereits im Jahr 1927 an das Vorkommen derartiger Infektionen in unserem Lande; er beobachtete „Bradsot" ähnliche Krankheitsfälle bei Schafen auf Terschelling. In diesen Fällen wurde aber keine Labmagen-, wohl eine Darmentzündung (Entero-toxaemie?, struck?) festgestellt. Weiter bemerkte Hibma (13), dass 12—24 Std. nach dem Tode die Nieren bereits breiig geworden waren; dies erinnert auch an „pulpy kidney" (wörtlich übersetzt: „Breiniere") eine Krankheit, die ebenfalls von *Cl. perfringens* verursacht wird. Hibma's Artikel weist darauf hin, dass bei Schafen derartige Anaëroben-Infektion auf Terschelling und vielleicht auch an anderen Orten des Landes vorkommen.

Zusammenfassung.

Aus einem Phlegmon der Magenwand eines Hundes wurde *Cl. perfringens* in Reinkultur isoliert. Aus dem Urin eines noch lebendigen Hundes und danach aus der Blase desselben getöteten Hundes, bei dem die Umgebung von Prostata und Blase phlegmonös entzündet war, wurde ebenfalls *Cl. perfringens* in Reinkultur isoliert; diese Kultur setzte Inulin nicht um, wohl Glycerol. Verf. vergleicht die Lokalisation in diesen Fällen mit bei anderen Tierarten bereits bekannten Infektionen von mit Schleimhäuten bekleideten Organen.

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IST ES MÖGLICH *LEPTOSPIRA ICTEROHAEMORRHAGIAE* DURCH KULTURVERFAHREN AUS OBERFLÄCHENWASSER ZU ISOLIEREN?

von

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(Eingegangen am 7 Juli 1941).

Es ist APPELMAN (2,3) im Jahre 1934 gelungen die Anwesenheit von *Leptospira icterohaemorrhagiae,* dem Erreger der WEILschen Krankheit, in künstlich oder natürlich infiziertem Oberflächenwasser nachzuweisen. Zu diesem Zweck badete er während einer Stunde Meerschweinchen mit rasierter und skarifizierter Bauchhaut im zu untersuchenden, bis zu 30°C. erwärmten, Wasser (je 3 Liter), das den Tieren bis an die Flanken reichte. Die Leptospiren drangen dann durch die verwundete Bauchhaut in das Tier hinein und machten es Weilkrank. Versuchserien mit künstlich infiziertem Leitungswasser zeigten, dass der Organismus aus Wasser isoliert werden kann, wenn sich wenigstens eine Leptospire in 3 ccm Wasser befindet. Bei der Verwendung von 10 Meerschweinchen wird dann sicher eins infiziert.

Die Bademethode APPELMANS hat der Praxis schon gute Dienste erwiesen. Mit Hilfe dieser Methode hat DAVIDSON (5) gezeigt, dass das frequente Vorkommen der WEILschen Krankheit unter Personen, die in Aberdeen Fische reinigen und verarbeiten, durch die Anwesenheit von Leptospiren verursacht wird, die sich auf dem Grunde in dem Abwasser befinden, in das Ratten uriniert haben.

Während der Mobilisierung habe ich zusammen mit Dr. W. L. C. VEER, der als Spezialoffizier des Militärpharmazeutischen Dienstes nach unserem Laboratorium zum Studium militärhygienischer Weilfragen detachiert war, die Frage studiert, ob es möglich wäre die APPELMANsche Methode zu verbessern.

Unter Hinweis auf die kurze Zusammenfassung unserer Arbeit (VAN THIEL und VEER (9)), teilen wir hier mit, dass es uns gelungen ist drei neue Methoden anzugeben, wodurch eine 8—17 Mal grössere Möglichkeit vorhanden war pathogene Leptospiren in Oberflächenwasser nachzuweisen, während die für den Versuch erforderliche Anzahl Meerschweinchen viel geringer ist.

Die erste Methode, der wir folgten, war eine „Zentrifugalmethode". In einer Zentrifuge vom Sharples-Typus der Firma Padberg werden 5 Liter Wasser mit einer Geschwindigkeit von 35000 Umdrehungen pro Minute zentrifugiert. Nachdem das Zentrifugat quantitativ in 25 ccm Wasser gesammelt ist, wird diese schlammige Flüssigkeit während 1.5 Stunden mit der rasierten und skarifizierten Bauchhaut von 2 Meerschweinchen in Berührung gebracht. Dazu wird ein Glaszylinder derartig auf den Bauch eines Tieres gebracht, dass die in den Zylinder hineingebrachte Flüssigkeit nicht herausfliessen kann. Obwohl mit dieser Methode die Möglichkeit besteht eine Leptospire in 20 ccm Wasser nachzuweisen, kann sie nicht empfohlen werden, weil sie einen teuren Apparat erfordert und weil die Meerschweinchen durch den Kontakt mit dem schlammigen Zentrifugat oft an einer Sepsis interkurrent sterben.

Die „Strommethode" kommt diesen Beschwerden entgegen. Nach dieser Methode lässt man das zu untersuchende Wasser ungefähr 2 Stunden langsam über die rasierte und skarifizierte Bauchhaut eines Meerschweinchens hinfliessen. Zu diesem Zweck wird wieder ein Glaszylinder auf der Bauchhaut befestigt, welcher dicht am unteren Rande eine Glastülle hat, durch welche das Wasser, das aus einem Heber in den Glaszylinder tropft, wieder hinausfliessen kann. Auf diese Weise werden ungefähr 200 ccm Wasser untersucht. Es ist nun sehr gut möglich eine Leptospire in 25 ccm, vielleicht sogar in 50 ccm, Wasser nachzuweisen. Mit dieser Methode gelang es einmal Weil-Leptospiren im „Uiterste Gracht" in Leiden zu finden, in welches ein Mann gefallen war, der kurze Zeit nachher Weilkrank wurde. Die Untersuchung geschah 4 Monate nach dem Fall ins Wasser.

Die dritte Methode ist die „subkutane Durchströmungsmethode". In die rasierte Bauchhaut eines Meerschweinchens wird ein 4 cm langer subkutaner Longitudinalkanal gemacht. An der einen Seite dieses Kanals ist eine Glasröhre befestigt, die mit einem Trichter, in welchem sich das zu untersuchende Wasser befindet, in Verbindung steht. An der anderen Seite des Kanals ist eine ungefähr 6 mm weite Öffnung, durch welche das unter der Haut durchströmende Wasser abfliessen kann. Die Strömungsgeschwindigkeit wird so gewählt, dass 100 ccm Wasser in 2 bis 2.5 Stunden durchfliessen. Mit dieser Methode ist die Möglichkeit, eine Leptospire in 50 ccm Wasser nachzuweisen, noch grösser geworden.

Die drei von uns beschriebenen Methoden können, ebenso wie die APPELMANsche Methode, „biologische" genannt werden. Alle brauchen das Meerschweinchen als Versuchstier. Dies befriedigt nicht. Erst dann kann man von einer guten Methode sprechen, wenn es gelingt, das Meerschweinchen völlig auszuschalten und die geringe Anzahl pathogener Leptospiren, welche in natürlich infiziertem Oberflächenwasser anwesend ist, sich derartig vermehren zu lassen, dass eine „kulturelle" Methode möglich geworden ist.

Appelman hat sich schon mit dieser Frage beschäftigt. Zwei Reihen von Isoliermethoden soll man unterscheiden: die erste führt zu stark bakteriell verunreinigten Leptospirenkulturen, die zweite versucht vom Anfang an eine Reinkultur von Leptospiren zu bekommen.

Erstgenannte Methode wurde u.a. von Zuelzer (12) in ihrem „Anreicherungsverfahren" angewandt. Unter Berücksichtigung der Tatsache, dass Leptospiren sich namentlich auf der Grenzfläche Schlamm-Wasser aufhalten, füllt sie eine Weckflasche (Inhalt 2 Liter) zum 4/5 Teile mit Schlamm oder mit Sand aus dem zu untersuchenden Graben oder Fluss, und füllt die Flasche bis an den Rand mit dem betreffenden Wasser. Nachdem der Schlamm oder der Sand sich abgelagert hat, werden Deckgläser auf die Oberfläche gelegt; darauf wird die Flasche mit Gummiring, Deckel und Klemme geschlossen. Die Leptospiren sammeln sich dann auf der oberen Seite der Deckgläser. Weiter konnte sie Kulturen der sich im Wasser befindenden Leptospiren bekommen, indem sie die Deckgläser in Leptospirenkulturmedien brachte.

Hindle (6) und Adamski (1) bringen das zu untersuchende Wasser (Leitungswasser) in Petrischalen, nachdem sie ein erbsengrosses Stückchen Kot von Mensch oder Tier hinzugefügt haben. Letztgenannter Untersucher giesst diese Flüssigkeit über eine Kotagarschicht aus.

Die Methoden Zuelzers und Hindles wurden von mir (mitgeteilt von Appelman (2, S. 67)) genau auf ihre Brauchbarkeit für die Isolierung von *L. icterohaemorrhagiae* aus Wasser geprüft. Dies geschah indem ich Kulturen dieser Spirochäte in natürliches Oberflächenwasser brachte; nachher versuchte ich diese Organismen nach beiden Methoden zurückzuzüchten. Wenn es gelang einen Leptospirenstamm zu züchten, dann war es immer *Leptospira biflexa* (*pseudoicterogenes*), der saprophytische Verwandte der Weil-Leptospiren ¹).

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¹) Nach dem Hinweis auf das wechselnde Resultat, das man mit dem Stuhlmedium nach Hindle und Adamski bekommt, erwähnt Zuelzer, dass die Anreicherung von Spirochäten ihr regelmässiger gelungen sei, wenn sie ausschliesslich menschliche Fäzes von Vegetariern für die Kulturen verwendete, vermutlich weil die Spirochäten schädigenden Eiweissabbauprodukte in diesen Fäzes nicht vorhanden sind.

Dr. Hulst, Assistent der internen Abteilung des Akademischen

Dies lässt sich auf den Umstand zurückführen, dass *L. icterohaemorrhagiae* schneller als *L. biflexa* im hier gebrauchten, stark durch Bakterien verunreinigten Milieu, stirbt. Man braucht sich also nicht zu wundern, dass wir einen stark skeptischen Standpunkt in Bezug auf die Möglichkeit, mittels beider Methoden *L. icterohaemorrhagiae* aus Oberflächenwasser zu isolieren, eingenommen haben.

Vinzent (10) und Bauer (4) folgen der zweiten Isoliermethode. Erstgenannter filtriert willkürliches Wasser durch Chamberland-Kerze L_5 und fügt dem Filtrat Serum hinzu. Letztgenannter filtriert nacheinander durch Berkefeld-Kerzen V und N und fügt dem Filtrat defibriniertes Meerschweinchenblut und Agar hinzu. Auf diese Weise bekommen sie Reinkulturen von Leptospiren.

Appelman hat mit Recht darauf hingewiesen, dass man in dieser Weise die besten Voraussichten hat, in Beziehung zu der relativen Seltenheit von Weil-Leptospiren, nur *L. biflexa* aus dem Wasser zu isolieren und im günstigsten Fall, wenn das Wasser also viele *L. icterohaemorrhagiae* enthält, im Filtrat sowohl *L. icterohaemorrhagiae* wie *L. biflexa* zu finden. Im günstigsten Fall bekommt man also endlich eine Mischkultur von *L. icterohaemorrhagiae* und *L. biflexa*.

Appelman hat nachgewiesen, dass die gleichzeitige Anwesenheit beider Organismen im von ihm verwendeten Korthof-Medium, mit der Verdrängung erstgenannter Leptospirenart endet. Auch in Bezug auf andere Leptospirenstämme und in verschiedenen flüssigen

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Krankenhauses in Leiden, hat mir Stuhl eines Patienten zur Verfügung gestellt, der schon während 5 Tage einer Diät von Gemüsen und Butter folgte.

50 Gramm dieser Fäzes wurde in 1 Liter Wasser sterilisiert und in flachen Kolben verteilt, die dann nacheinander mit 1, 3, 5, 10 Tropfen und 2 ccm *L. icterohaemorrhagiae*-Kultur geimpft wurden. Nur im mit 2 ccm Kultur (in diesem Kolben war also relativ mehr Serum als in den anderen) geimpften Kolben entstand eine reiche Kultur; die anderen Kolben blieben negativ.

Weiter brachte ich ein wenig derselben nicht-sterilisierten Fäzes in Petrischalen (nach Hindle), in welchen sich Grabenwasser, Grachtenwasser oder Leitungswasser, sterilisiert oder nicht, befand. Zum Inhalt jeder Schale wurden 3 Tropfen *L. icterohaemorrhagiae*-Kultur hinzugefügt. Nur in den mit Leitungswasser versehenen Schalen entstand Wachstum von Leptospiren; die übrigen Schalen blieben negativ.

Die Ergebnisse blieben unter die Erwartung, so dass die Verwendung von Vegetarierfäzes für die Isolierung von pathogenen Leptospiren aus Wasser sich nicht empfehlen lässt.

Kulturmedien habe ich bestätigen können, dass beide Organismen sich tatsächlich als Antagonisten benehmen [1]).

Mit Hilfe der Filtrationsmethode nach VINZENT (indem BAUER dem Filtrat Agar hinzufügt, gleicht seine Methode mehr der nachher zu besprechenden Eiagarmethode nach ZUELZER) konnte der Erreger der WEILschen Krankheit nur dann nachgewiesen werden, wenn das Filtrat sofort nach der Filtrierung bei Versuchstieren eingespritzt wurde. Eine morphologisch differentielle Untersuchung der genannten Leptospiren ist ja unmöglich, weil beide Organismen einander völlig gleichen. Man würde aber sehr viele Meerschweinchen nötig haben, um alle Filtrierflüssigkeit einspritzen zu können. Mittels Zentrifugieren ist es noch nicht möglich einige wenige Leptospiren aus einer relativ grossen Quantität Wasser zu isolieren.

Unter wird untersucht werden, ob mit der Filtriermethode, wenn man sie mit der näher zu besprechenden kulturellen Methode nach ZUELZER kombiniert, etwas erreicht werden kann.

Wenn man sich diese Tatsachen überlegt, so ist es klar, dass die in der Überschrift dieser Abhandlung gestellte Frage verneint werden muss.

Nun aber hat ZUELZER (13) vor einigen Jahren einige Bemerkungen über den soeben beschriebenen Antagonismus gemacht. Weder biologische Erfahrungen, noch das Benehmen der Spirochäten in der freien Natur, noch die Beobachtungen aus der Praxis über den Zustand des Wassers, in welchem Weilinfektionen zustande kommen, stützten — nach ihr — unsere Beobachtungen. Statt unsere Versuche zu wiederholen hat sie aber unseren Schlüssen eine Interpretation gegeben, die wir selbst nicht hineingelegt haben. Nirgendwo haben wir ja behauptet, dass für die freie Natur dasselbe gilt wie für das Reagenzgläschen.

Dass der Antagonismus zwischen apathogenen saprophytischen und pathogenen Leptospiren nicht bestehen würde, gründet sie weiter auf die folgenden Versuche.

Auf einen in einer Petrischale gegossenen Eiagarnährboden, in welchem beide Leptospirearten gut wachsen können, impfte sie *L. biflexa*-Reinkultur aus Serumwasser. Nachdem die Leptospiren sich soweit vermehrt hatten, dass sie in jedem untersuchten Präparat auf der ganzen Platte gefunden wurden, wurde 1.5 ccm einer virulenten Leptospiren-

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[1]) Nach der Ursache dieses Antagonismus, für den ich auf Grund meiner Experimente die Absonderung durch *L. icterohaemorrhagiae* von der *L. biflexa* schädlichen Stoffen verantwortlich machte, soll eine nähere Untersuchung angestellt werden.

Reinkultur auf die Platte gebracht. Bei der Impfung des Plattenmaterials bei Meerschweinchen nach 20 Tagen zeigte sich, dass der pathogene Stamm noch in unverändertem Zustande anwesend war.

Dasselbe Ergebnis erhielt sie in zwei Versuchsserien in welchen die Eiagarplatte sofort mit saprophytische Leptospiren enthaltendem Leitungswasser angefertigt wurde. Als ein reiches Wachstum dieser Leptospiren neben einem solchen von Bakterien und Flagellaten entstanden war, impfte sie in einem Versuch 1 ccm Kultur eines pathogenen Stammes und in einem anderen virulente Leptospiren enthaltenden Rattenurin. Nach 10 Tagen wurde Plattenmaterial mit positivem Resultat bei Meerschweinchen geimpft.

Zum Schluss teilt sie noch mit, dass SCHÜFFNER letztgenannten Versuch wiederholte mit *Leptospira canicola* enthaltendem Hundeurin. Alle 5 Tage impfte er während eines Monates Meerschweinchen mit Material von der Platte. Alle geimpften Tiere erkrankten unter dem typischen Bilde der WEILschen Krankheit (In einem späteren Versuch gelang es ihm nicht auf diese Weise einen ähnlichen, schwach virulenten Stamm aus Hundeurin zu züchten, weil die Begleitbakterien die Spirochäten überwucherten).

Diese Versuche zeigen tatsächlich, dass pathogene Leptospiren unter Umständen, die in der Eiagarplatte herrschen, in Mischkulturen mit *L. biflexa* und anderen Organismen nicht verdrängt werden. Ein jeder wird aber sofort zugeben müssen, dass es einen grossen Unterschied gibt zwischen den Versuchen ZUELZERS und denjenigen von APPELMAN und mir. In den erstgenannten Versuchen müssen die Leptospiren sich im Agar bohrend fortbewegen, in den unsrigen dagegen besteht eine völlig freie Fortbewegung im flüssigen KORTHOF-Medium. Jedenfalls hat ZUELZER nicht das Recht den von uns konstatierten Antagonismus kraft ihrer Experimente in Zweifel zu ziehen.

Es fragt sich jetzt, ob der Eiagarnährboden ZUELZERS für die Isolierung von *L. icterohaemorrhagiae* aus Oberflächenwasser Wert hat. ZUELZER selbst sagt, dass dieser Nährboden ausgezeichnet ist ,,sowohl zum Nachweis vereinzelter Spirochäten aus reinem Wasser, als auch zur Isolierung von Spirochäten aus belebtem Wasser....”. Die Spirochäten würden in dem Nährboden zusammen mit Protozoen, Protophyten und Bakterien, die in der Biocoenose vorkommen, wachsen. Nirgendwo hat sie aber gezeigt, dass sie auf diese Weise *L. icterohaemorrhagiae*, die in einigen Exemplaren in Oberflächenwasser anwesend ist, daraus isolieren kann. Wohl hat sie gezeigt, dass Platten positiv werden, die mit Wasser geimpft werden (respektive mit Wasser gemacht werden), in welchem zuvor keine Leptospiren entdeckt werden können. Ein Unterschied zwischen pathogenen und saprophytischen Stämmen wird dabei nicht gemacht. Auch kann man aus ihren Experimenten, die den von uns beschriebenen Antagonismus zwischen pathogenen und saprophytischen Leptospiren widerlegen müssten, nicht schliessen, dass die Isolierung einer einzigen pathogenen Leptospire aus „belebtem Wasser” mittels ihrer Methode möglich ist.

Meine Untersuchung hat sich mit den folgenden Fragen beschäftigt:

a. Tritt der Antagonismus zwischen *L. icterohaemorrhagiae* und *L. biflexa* bei der Verwendung des Eiagarnährbodens nach ZUELZER tatsächlich nicht auf? Ist das der Fall, dann könnte dieser Nährboden gute Dienste für die Beantwortung der folgenden Frage erweisen.

b. Ist es möglich die Anwesenheit einzelner Exemplare von *L. icterohaemorrhagiae* in Oberflächenwasser mit Hilfe des Eiagarnährbodens nachzuweisen?

c. Kann diese Anwesenheit vielleicht durch die Kombination dieser Kulturmethode mit der Filtriermethode nach VINZENT nachgewiesen werden?

a. Der Antagonismus zwischen L. icterohaemorrhagiae und L. biflexa im Eiagarnährboden nach ZUELZER [1]).

Der Nährboden wurde in folgender Zusammensetzung benutzt: 2 gr. Agar, 225 ccm Wasser, 75 ccm Phosphatpufferlösung in Aqua redest.

Der pH des Nährbodens wurde dem pH des zu untersuchenden Wassers gleichgemacht (meistens pH 7.4).

Nach der Sterilisierung lässt man die Flüssigkeit auf 30°—40°C. abkühlen und fügt 1 ccm Eigelb und 3 mgr Brilliantgrün hinzu. Dann giesst man die Flüssigkeit in grosse Petrischalen aus. Nach der Beimpfung werden sie in einen Brutschrank von ungefähr 24°C. gebracht.

Weil es von Wichtigkeit war zu erfahren, ob der Antagonismus im Nährboden auftrat, wenn man mit diesem Medium Leptospiren aus Oberflächenwasser isolieren wollte, wurden die Versuche nicht mit Reinkulturen gemacht. Im zweiten und dritten obengenannten Versuch ZUELZERS geschah dies ebensowenig. In unseren früheren Versuchsreihen, wobei der Antagonismus festgestellt wurde, war dies wohl nötig, weil wir studieren wollten was geschehen würde, wenn beide Leptospirearten nach der Filtrierung, also ohne gleichzeitige Anwesenheit von Bakterien, im üblichen Nährboden zusammen wären.

Meine Versuchsaufstellung war insofern anders als bei ZUELZER, weil von mir je 30 ccm frisches Grabenwasser, dem mehrere Mengen Spirochäten besagter Art hinzugefügt worden waren, auf die Platten gebracht wurde. ZUELZER brachte ja nur 0.5—1.5 ccm Wasser darauf. Für die Untersuchung des Oberflächenwassers ist dies eine zu kleine Quantität Wasser als dass man je pathogene Leptospiren daraus isolieren könnte. Diese grössere Quantität Wasser schien mir keine Schwierigkeit, weil ZUELZER selbst feststellt, dass die Spirochäten in den Agar hineindringen, wodurch die Organismen sich dann meiner Voraussetzung zufolge antagonistischen Einflüssen entziehen könnten.

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[1]) Die Verwendung von Eiagar bei der Leptospirenzucht ist schon von WALKER (11) im Jahre 1927 empfohlen worden.

Drei grosse Platten wurden zu gleicher Zeit auf die folgende Weise geimpft:

Platte I mit 30 ccm frischem klarem Grabenwasser, dem 0.5 ccm Reinkultur von *L. biflexa* und 1 ccm virulente Reinkultur von *L. icterohaemorrhagiae* hinzugefügt worden war.

Platte II mit 30 ccm frischem klarem Grabenwasser, dem 0.5 ccm Reinkultur von *L. biflexa* hinzugefügt worden war. Nach einer Woche, als zahlreiche Leptospiren sowohl im Agar als in der obenstehenden Flüssigkeit anwesend waren, wurde 1.5 ccm virulente Reinkultur von *L. icterohaemorrhagiae* hinzugefügt.

Platte III mit 30 ccm frischem klarem Grabenwasser. Als es sich zeigte, dass nur wenige Leptospiren nach einer Woche anwesend waren, wurde noch eine Öse *L. biflexa* hinzugefügt. Als nun nach vier Tagen mehr Leptospiren gefunden wurden, wurden 5 Tropfen virulente *L. icterohaemorrhagiae* hinzugefügt.

Etwa einmal in der Woche wurde ein Teil der Flüssigkeit (0.5—1 ccm) und zugleicherzeit je ein Blöckchen (1 qcm Oberfläche) zentral und peripher aus dem Agar genommen und bei Meerschweinchen subkutan eingespritzt, nachdem das Material auf die Anwesenheit von lebenden Leptospiren untersucht worden war.

In der Platte I fanden die Leptospiren sich überall, bis ans Ende des Versuchs, in ausserordentlich starkem Wachstum. In der Platte II war ihre Anzahl viel geringer; sogar nach 24 Tagen [1]) gab es wenig lebendige Leptospiren in der Flüssigkeit.

In der Platte III. enthielt ein Teil des Materials bis nach 24 Tagen viele Leptospiren, nach 31 Tagen aber viel weniger; bis ans Ende des Versuchs wurden dann selten mehr lebendige Spirochäten gesehen.

Studiert man die in der Tabelle I zusammengefassten Resultate, so ergibt sich Folgendes:

1. In der Platte III haben wir ein Resultat, das mit dem im von SCHÜFFNER gemachten Versuch übereinstimmt. Statt eines Monats blieben die pathogenen Leptospiren hier sogar während 83 Tage

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[1]) Es handelt sich jedesmal um die Tage nach der Hinzufügung der virulenten Leptospiren.

Tabelle I.

Nummer der Platte und nähere Einzelheiten	Anzahl Tage nach der Hinzufügung der virulenten Leptospiren	Einspritzung bei Meerschweinchen mit folgenden Teilen der Platte:					
		Obenstehende Flüssigkeit		Zentrum des Agars		Peripherie des Agars	
		Leptospirenmenge	Resultat Meerschweinchenversuch	Leptospirenmenge	Resultat Meerschweinchenversuch	Leptospirenmenge	Resultat Meerschweinchenversuch
I. Grabenwasser + 0.5 ccm *L. biflexa* + 1 ccm virulente *L. icterohaemorrhagiae*	7 Tage	+++	pos.	+++	pos.	+++	pos.
	14 ,,	+++	neg.	+++	neg.	+++	neg.
	24 ,,	+++	neg.	+++	neg.	+++	neg.
	31 ,,	+++	neg.	+++	†	+++	neg.
	38 ,,	+++	†	+++	neg.	+++	neg.
II. Grabenwasser + 0.5 ccm *L. biflexa* + (nach einer Woche) 1.5 ccm virulente *L. icterohaemorrhagiae*	7 ,,	+++	pos.	++	pos.	++	pos.
	17 ,,	++	pos.	++	pos.	++	pos.
	24 ,,	+	pos.	++	pos.	+	pos.
	31 ,,	+	†	+	neg.	—	†
	38 ,,	+	neg.	+	†	+	neg.
III. Grabenwasser + (nach einer Woche) 1 Öse *L. biflexa* + (nach 4 Tagen) 5 Tropfen virulente *L. icterohaemorrhagiae*	10 ,,	++	pos.	++	pos.	++	pos.
	17 ,,	+++	pos.	++	pos.	+++	pos.
	24 ,,	+	†	+++	†	++	pos.
	31 ,,	+	pos.	+	pos.	+	pos.
	40 ,,	—	pos.	+	neg.	+	pos.
	53 ,,	—	pos.	(+)	pos.	+	pos.
	70 ,,	—	pos.	(+)	†	(+)	†
	83 ,,	—	neg.	—	pos.	—	pos.

+++ = reiches Leptospirenwachstum
++ = viele Leptospiren
— = keine Leptospiren
+ = einzelne Leptospiren
(+) = nicht sicher ob die einzelnen Leptospiren lebendig sind
† = interkurrent tot

in virulentem Zustande anwesend. Hier hat sich also nichts von einem Antagonismus zwischen *L. biflexa* und *L. icterohaemorrhagiae* gezeigt.

2. In der Platte I dagegen, in welcher dieselben Leptospirestämme als in der Platte III geimpft wurden, ist dieser Antagonismus klar zum Ausdruck gekommen; in der Platte II aber in weniger starkem Masse.

3. Die mikroskopische Untersuchung des beim Meerschweinchen geimpften Materials auf die Anwesenheit von Leptospiren gibt die Erklärung des in den drei Platten erhaltenen Resultats.

In der Platte I war ein so starkes Leptospirenwachstum, dass man leicht annehmen konnte, dass *L. biflexa* dabei in so stärker Frequenz anwesend sei, dass der Einfluss des Antagonismus leicht zum Ausdruck kommen könnte und zwar entweder sowohl in der Flüssigkeit wie im Agar, oder nur in der Flüssigkeit. Im letzteren Fall müsste man annehmen, dass die pathogenen Leptospiren nicht die Neigung haben in den Agar hineinzudringen. Für diese Voraussetzung gibt es aber keinen Grund, weil die pathogenen Leptospiren sich im selben Versuch nach 7 Tagen wohl im Agar fanden und der Versuch mit der Platte III deutlich zeigt, dass sie sich in den Agar hineinbohren.

In der Platte III dagegen gab es nach einer Woche, ungeachtet der Ursache, ein so geringes Leptospirenwachstum, dass eine Öse *L. biflexa* nachgeimpft werden musste um ein stärkeres Wachstum dieser Organismen zu bekommen. Man muss annehmen, dass das Wachstum von *L. biflexa* auch nachher nicht so kräftig gewesen ist wie in der Platte I, denn sonst hätte ich ein viel stärkeres Wachstum von Leptospiren feststellen müssen. Es ist also hier für *L. icterohaemorrhagiae*, sowohl in der Flüssigkeit wie im Agar, in jeder Hinsicht möglich gewesen sich den antagonistischen Einflüssen zu entziehen.

Dass das Resultat in der Platte II die Mitte hielt zwischen den Resultaten der beiden anderen Platten, lässt sich aus der, im Vergleich mit der Platte I, geringeren Anzahl Leptospiren und der grösseren Menge der hinzugefügten pathogenen Leptospiren erklären.

4. Es hängt völlig von den Umständen ab, ob der Antagonismus zwischen *L. biflexa* und *L. icterohaemorrhagiae* im Eiagarnährboden zum Ausdruck kommt.

Dass dies nicht der Fall war in den Versuchen ZUELZERS und in denen SCHÜFFNERS, erkläre ich dadurch, dass diese beiden Untersucher nur wenig (nacheinander 1.5 ccm und 1 ccm Reinkultur, einige Tropfen Rattenurin) pathogene Leptospiren enthaltende Flüssigkeit auf die Platten impften, auf welche vorher wenig (1.5 ccm) oder kein (die Platte ist dann direkt mit *L. biflexa* enthaltendem Wasser gegossen; sieh unten) *L. biflexa* enthaltendes Wasser gebracht wurde. Es lässt sich denken, dass die geringere Durchtränkung des Agars mit Wasser eine günstigere Gelegenheit für *L. icterohaemorrhagiae* bot sich im Agar zu behaupten als in meinen Versuchen. ZUELZER hat nachdrücklich erwähnt, dass die Spirochäten in der oberen Agarschicht und nicht in der sich darüber befindenden Flüssigkeit gefunden wurden.

5. Es ist nicht ausgeschlossen, dass der Eiagarnährboden bei epidemiologischen Studien der WEILschen Krankheit gute Dienste erweisen kann.

b. Der Eiagarnährboden nach ZUELZER *bei der Isolierung von L. icterohaemorrhagiae aus Oberflächenwasser, in welchem sich nur einige Exemplare dieses Organismus befinden.*

ZUELZER hat angegeben, dass im Falle der Untersuchung reines, bakterienarmes Wassers und der Nachweisung von ,,sehr spärlichem Spirochätenmaterials'' das betreffende Wasser direkt für die Anfertigung des Nährbodens verwendet werden kann.

Nachdem das Agarpuffergemisch auf 30—40°C. abgekühlt ist, wird es mit diesem auf 20°C. erwärmten Wasser, dem Eigelb hinzugefügt worden ist, gemischt und auf Platten verteilt.

Zur Beantwortung der Frage, ob diese Methode in der Praxis der Weiluntersuchungen angewendet werden könne, wurde Wasser untersucht, das zwar nicht ,,reines bakterienarmes Wasser'' zu sein beansprucht, aber das doch sehr klar war und in welchem oft geschwommen wird. Diesem Wasser wurden nun einige virulente Spirochäten hinzugefügt. Um die Aussicht auf ein günstiges Resultat so gross wie möglich zu machen, wurde dieses ,,einige'' etwas ,,weit'' aufgefasst. Also wurden 46 ccm Wasser 20 Exemplare hinzugefügt. Von diesem Wasser wurden nun 5 kleine Platten (I—V) nach der soeben beschriebenen Methode gegossen. Sie wurden im Dunkeln bei einer Temperatur von ungefähr 24°C. erhalten.

9—11 Tage nach der Einstellung des Versuchs wurden Muster von 16 Teilen jeder Platte (von jedem Quadranten 3 Teile peripher parallel mit dem Rande und ein Teil dicht beim Mittelpunkt der Platte) genommen und auf die Anwesenheit lebender Leptospiren untersucht. Aus den Platten wurden dann verschiedene Quantitäten Agar subkutan bei Meerschweinchen eingespritzt.

In der Platte I zeigte sich der periphere Teil von $1^2/_3$ Quadranten positiv. Diese ganze Menge wurde beim Meerschweinchen Nr. 1, das 5 Tage nachher einer Sepsis zufolge starb, eingespritzt.

In der Platte II waren alle Muster, mit Ausnahme von 3 peripheren, positiv. Beim Meerschweinchen Nr. 2 wurde ein Wenig der Zentralpartie eines Quadranten, in welchem weniger Leptospiren waren als in anderen Teilen, subkutan beim Meerschweinchen Nr. 2 eingespritzt. Auch dieses starb infolge einer Sepsis, nach 7 Tagen.

Aus derselben Platte wurden Muster der positiven Teile beim Meerschweinchen Nr. 3 eingespritzt. Dieses Tier starb nach 10 Tagen, auch einer Sepsis zufolge; das Blut enthielt keine Antikörper gegen *L. icterohaemorrhagiae*.

In der Platte III waren die 7 positiven und die übrigen negativen Teile sehr unregelmässig über die Platte verteilt. Aus allen positiven Teilen wurde ein Muster genommen und subkutan beim Meerschweinchen Nr. 4 geimpft. Das Tier erkrankte nicht; das Blut enthielt keine Agglutininen, respektive Lysinen gegen *L. icterohaemorrhagiae* 20 Tage nach der Impfung.

In der Platte IV enthielten nur 5 periphere Muster keine Leptospiren. Aus allen positiven Teilen wurde ein Muster und wie zuvor beim Meerschweinchen Nr. 5 eingespritzt. Auch dieses erkrankte nicht; keine Infektion mit *L. icterohaemorrhagiae* fand statt.

In der Platte V war die eine Hälfte, mit Ausnahme von 2 peripheren Teilen, positiv, die andere völlig negativ. Muster der positiven Teile wurden beim Meerschweinchen Nr. 6 eingespritzt. Das Tier bekam keine Temperaturerhöhung und wurde nicht Weilkrank.

Dieses Resultat kann völlig enttäuschend genannt werden. Drei Meerschweinchen starben zufolge einer Sepsis mit ausgedehnten subkutanen Abszessen, in dem Augenblick, wo die WEILsche Krankheit noch nicht festgestellt werden konnte; drei blieben gesund. Obwohl also Muster aus verschiedenen, lebende Leptospiren enthaltenden Teilen der Platte untersucht wurden, sind diese Leptospiren wenigstens bei der Hälfte der Versuche (3 der 6 Meerschweinchen blieben ja gesund) *L. biflexa*-Kolonien gewesen. Es war also nicht möglich, *L. icterohaemorrhagiae* zurückzufinden. Wir lassen es dahingestellt sein, ob dies dem Antagonismus zwischen *L. icterohaemorrhagiae* und *L. biflexa* oder dem Einfluss der zahlreichen Bakterien, Protozoen und Protophyten zufolge entstanden ist. Sicher ist dass der Bakterienreichtum des Wassers den Sepsistod der Meerschweinchen verursacht hat.

Um diese Methode in der Praxis mit gutem Erfolg anwenden zu können, soll, der Meinung ZUELZERS entsprechend, bakterienarmes Wasser verwendet werden. Als solches verwendete sie Leitungswasser. Es ist aber klar, dass die in diesem Falle erhaltenen guten Resultate für die Untersuchung des Oberflächenwassers auf die Anwesenheit von *L. icterohaemorrhagiae* keine Bedeutung haben.

Wenn man nun bedenkt, dass es mit der von uns beschriebenen (siehe oben) „Strommethode" und „subkutanen Durchströmungsmethode" gewiss gelungen wäre die Infektion dieses Wassers mit nur einem Meerschweinchen nachzuweisen, dann liegt der Schluss auf der Hand, dass ich vom Eiagarnährboden für das epidemiologische Studium der WEILschen Krankheit vorläufig nicht viel Gutes erwarte.

Dieser Schluss gilt *mutatis mutandis* auch für die in der Einleitung genannte Methode Bauers.

c. Die Filtriermethode nach Vinzent, *kombiniert mit der Eiagarnährbodenmethode nach* Zuelzer *bei der Isolierung von L. icterohaemorrhagiae aus Oberflächenwasser, in welchem sich nur wenige Exemplare dieses Organismus finden.*

Weil der Eiagarnährboden eine Möglichkeit an die Hand gibt den mehrerwähnten Antagonismus nicht stattfinden zu lassen (siehe unter *b*), habe ich vom nach der Filtriermethode Vinzents bekommenen Filtrat sofort Eiagarplatten gegossen, um dem Auftreten des Antagonismus, der gemäss dem in der Einleitung Erwähnten dem Erreichen eines günstigen Resultats im Wege steht, vorzubeugen.

Weil mir nur Chamberland-Kerzen (L_2 und L_3) zur Verfügung standen, habe ich die Bauersche Methode nicht angewandt. Dies gibt keine Schwierigkeiten, weil die Prinzipien beider Methoden dieselben sind. Die Versuche habe ich in der Tabelle II erwähnt.

50 ccm klarem Oberflächenwasser wurden wenige pathogene und wenige saprophytische Leptospiren zugefügt. Dieses infizierte Wasser wurde weiter durch eine L_2-Kerze filtriert. Eine selbe Quantität, auf dieselbe Weise infiziertes, Wasser wurde durch eine L_3-Kerze filtriert. Die Filtrate wurden je mit sterilem Leitungswasser bis zu 46 ccm angefüllt, erwärmt bis 30°C. (es zeigte sich dass 20°C. zu niedrig ist) und mit dem folgenden auf 30—40°C. abgekühlten Eiagarnährboden gemischt:

10 ccm 2.5% Agar in Leitungswasser, 19 ccm Phosphatpufferlösung pH 7.4, 0.25 ccm Eigelb (nach Abkühlung auf 30—40°C.).

Von diesem Gemisch wurden 5 Platten gegossen. Nach 10, 20 und 30 Tagen wurden die Platten mikroskopisch auf die Anwesenheit von Leptospiren kontrolliert; im dritten Versuch wurde die Hälfte des Agars aus jeder Platte bei Meerschweinchen (zwei mit L_2-Filtratplatten und zwei mit L_3-Filtratplatten) subkutan eingespritzt.

In den Versuchen 3 und 4 wurde jedesmal von 40 ccm Wasser ausgegangen, weil jedesmal noch 10 ccm Wasser in die Kerzen gebracht wurde, nachdem das übrige Wasser filtriert war. Diese Methode habe ich immer mit gutem Erfolg angewandt (van Thiel (7)), um die in den Kerzenporen zurückgebliebenen Leptospiren doch noch zu zwingen ins Filtrat hineinzugehen. Dies wurde durch die Erhöhung des Filtrationsdrucks noch gefördert.

Ungeachtet der Tatsache, dass dem Wasser also wenigstens 20 Mal soviel Leptospiren hinzugefügt wurden (d.h. mehr Leptospiren als wahrscheinlich jemals in Oberflächenwasser anwesend sein können) als wir mit unseren biologischen Methoden aus Wasser isolieren konnten, lehrt die Tabelle II, dass es nicht möglich gewesen ist, ihre An-

Tabelle II.

Nummer des Versuchs	Anzahl der den 50 ccm Wasser hinzugefügten Leptospiren		Filtrationsdruck in cm Quecksilber		Resultat in Agarplatten			
					L_2-Filtrat		L_3-Filtrat	
	L. icterohaemorrhagiae	*L. biflexa*	Erster Teil der Filtration	Zweiter Teil der Filtration.	Anwesenheit von Leptospiren	Meerschweinchenversuch	Anwesenheit von Leptospiren	Meerschweinchenversuch
1	20	10	70	70	neg.	—	neg.	—
2	20	20	70	70	neg.	—	neg.	—
3	20	20	20	70	neg.	neg.	neg.	neg.
4	20	20	18	18	neg.	—	neg.	—

wesenheit auf die beschriebene Weise nachzuweisen. Dies wundert nicht, weil die Anzahl Leptospiren, die durch eine Filterkerze hindurchgeht, im Vergleich zu der Anzahl die sich in der zu filtrierenden Flüssigkeit befindet, doch nur ausserordentlich gering ist. Mit einer L_5-Kerze, wie sie von VINZENT benutzt wurde, würde das Resultat gewiss negativ gewesen sein.

Es ist also nicht möglich durch die Kombination der Filtriermethode nach VINZENT mit der Kulturmethode nach ZUELZER *L. icterohaemorrhagiae* aus Oberflächenwasser zu isolieren.

Zusammenfassung.

1. Weil der von APPELMAN und VAN THIEL beschriebene Antagonismus zwischen der pathogenen *Leptospira icterohaemorrhagiae* und der saprophytischen *Leptospira biflexa* im Eiagarnährboden nach ZUELZER unter bestimmten Umständen nicht zum Ausdruck kommt, habe ich versucht mit Hilfe dieses Nährbodens *L. icterohaemorrhagiae* aus Oberflächenwasser, in welchem sich nur wenige Exemplare dieser Spirochäte befinden, zu isolieren.
2. Obgleich zahlreiche virulente Leptospiren dem zu untersuchenden Wasser hinzugefügt wurden, hat es sich als nicht möglich erwiesen ihre Anwesenheit durch die Injektion des kultivierten Materials bei Meerschweinchen nachzuweisen.
3. Auch durch die Kombination der Filtriermethode VINZENTs mit der Eiagarmethode ZUELZERs ist dies nicht möglich gewesen.
4. Weil *L. icterohaemorrhagiae* durch Zuchtverfahren noch nicht aus Oberflächenwasser isoliert werden kann, ist die Anwendung der von uns beschriebenen Methoden notwendig.

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ON THE ACTION OF SULFANILAMIDE

IV. IS THE SULFANILAMIDE MOLECULE ALTERED BEFORE ACTION? [1]

by

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1. INTRODUCTION.

In a previous communication (9) on the subject we determined with an adequate technique the number of viable bacteria as a function of the time, working with sulfanilamide containing cultures of *Streptococcus haemolyticus* in blood broth medium.

When inocula have been small and the cultures are fast growing sulfanilamide induces a characteristic growth curve. Its shape differs from the curves obtained with any other germicide (phenol, trypaflavine). The action of sulfanilamide is delayed for some hours, growth during this lag time of action being equal to that of the control culture. This rather striking phenomenon, in our opinion the best characteristic of the drugs of the sulfanilamide group, was observed and studied too by other investigators (see f.i. 1, 2, 6). Besides MAYER (7) claims sulfanilamide to be only a precursory substance for the actual antibacterial product. He supposes the drug to be altered by a process of oxidation. The same hypothesis is held up by FOX (2). Also LOCKE and co-workers (5, 8), supposing that the mechanism of sulfanilamide action is due to catalase inactivation, assume the transformation of the substance into an active product, probably by oxidation, of the type of hydroxylamine. For an analysis of this hypothesis see (4).

The relative low toxicity and the special properties of the remarkable drug seem to furnish some objections to the assumption, that it acts qua talis. This must be the reason why the assumption that the original substance is transformed into some active molecule finds general favour.

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[1] Third communication: H. W. JULIUS and A. SALOMON, Antonie van Leeuwenhoek **7**, 77, 1941.

In this communication we have tried to answer this fundamental question. Without a sure basis in this respect it has no sense to make suppositions in which direction a transformation would be performed.

2. METHOD.

There are two fundamentally different ways along which the problem might be approached.

a. A suitable medium is to be inoculated with a suitable, *i.e.* a ,,small'' number of bacteria (streptococci) and sulfanilamide added to make a suitable concentration (f.i. $0.15^0/_{00}$). After some time the sulfanilamide is to be ,,taken out'' of the medium and to be substituted by a new amount to the same concentration. If the drug needs to be transformed before it may display its action the manifestation of its antibacterial activity must be delayed for as long a period as the first situation has lasted. If however the newly added genuine sulfanilamide is able to continue the process such as if the first portion was still there, it may be concluded that transformation of the substance does not occur, or at least that this is not essential. Of course it has, as a control, to be proved that a removal of the substance without a substitution by another amount re-establishes the growth potencies of the cocci at once.

b. A suitable medium is to be inoculated as in case *a.* After some time the cocci are to be taken out and they are to be substituted by a new inoculum. If the substance is transformed in the preceeding period the curve must follow the same course as it would have done without substitution of the bacteria. If however the substance as such is active the newly added cocci will behave as if they came freshly in the unaltered sulfanilamide; *i.e.* there will be the lag time of sulfanilamide action in the curve as usual.

After these theoretical considerations we came to the following technique and results.

3. TECHNIQUE AND RESULTS.

Using a 50% blood broth medium and streptococci as the organism, it is clearly impossible to fulfill the postulations mentioned, at least if we take them to the letter. But in the following way we succeeded to approach them very nearly. The counting method described previously (3,9) which allows the construction of a curve of the process, enables us to work quantitatively in a rather exact way. Thus, ,,taking away" the sulfanilamide can be realised by diluting the sulfanilamide containing media at any moment of the process over a known range f.i. 1 : 100. On the other hand ,,taking away" the bacteria is impossible, but it is very well possible to add a suitable number of fresh bacteria to the original medium and to calculate what would be the resulting curve, if the newly added cocci would find a medium, ready to act on them (sulfanilamide altered) and to calculate, on the other hand the shape of the curve, if the second inoculum had to pass through the same lag time as the initial one (sulfanilamide not altered).

The following three experiments were taken and repeated some times with the same results:

A. To exclude the possibility that the sulfanilamide drug might be altered in some way by the blood (which forms part of the medium), the substance has been added to a concentration of 0.015% 18,3,2 and 1 hours before and also at the moment of inoculation with streptococci.

Fig. 1 indicates, that there is no influence whatever on the activity under the conditions of the experiments. So we may conclude, that there is no interaction of the blood and the drug, at least not in such a way, that the character of the antibacterial activity is altered. The phenomenon of lag time can not be explained by any such alteration.

B. Two tubes with 5 ml of 50% blood broth medium were inoculated with a suitable amount of streptococci. To one of them sulfanilamide was added in an amount to make a concentration of 0.015%. Growth curves from hourly determinations were constructed. Two and three hours after the addition of the drug volumes of two standard drops were taken from both tubes and brought into a hunderdfold volume of the medium. The drops taken out of the control culture were supplied to the same medium. Those taken out of the sulfanilamide containing culture were conveyed to a) a medium with and to b) a medium without sulfanilamide.

These cultures, too, furnished data to construct growth curves with. The results can be read from Fig. 2; the following conclusions may be drawn:

a. that the technique is reliable. The curves of the original and sub-inoculated control cultures run strictly parallel with a difference of 2 in the logarithm.

b. that the dilution of 1 : 100 of this concentration is nearly suf-

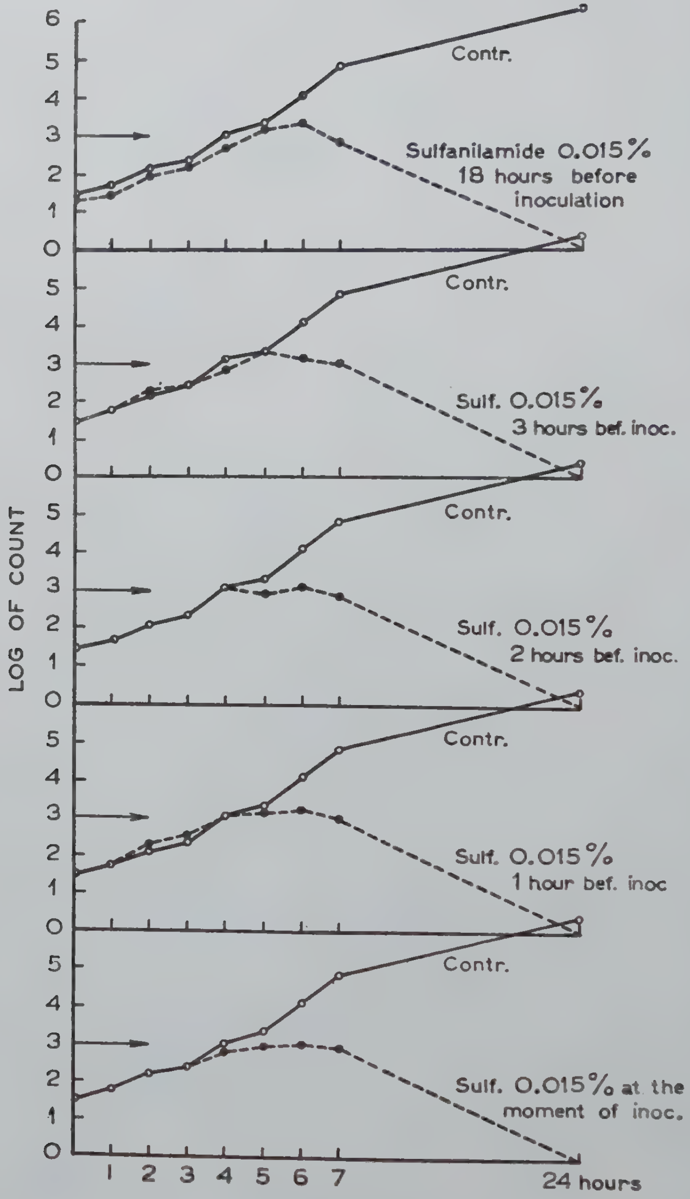
LOG OF COUNT
6
5
4
3
2
1
0
Contr.
Sulfanilamide 0.015 % 18 hours before inoculation
Contr.
Sulf. 0.015 % 3 hours bef. inoc.
Contr.
Sulf. 0.015 % 2 hours bef. inoc.
Contr.
Sulf. 0.015 % 1 hour bef. inoc
Contr.
Sulf. 0.015 % at the moment of inoc.
1 2 3 4 5 6 7
24 hours

Fig. 1.

ficient to annihilate the influence of sulfanilamide. The cocci are able to grow at the same rate as the controls.

c. that the cocci brought into a fresh solution of sulfanilamide behave

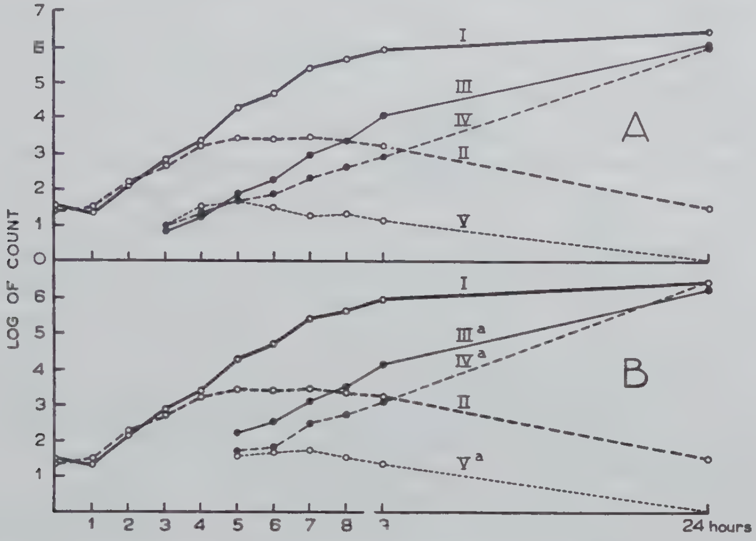

Fig. 2. Group A after 3 hours; Group B after 5 hours.
I = Control (Streptococci in 50% blood broth medium).
II = Sulfanilamide 0.015%.
III = Inoculation 1 : 100 from I in control medium.
IV = Inoculation 1 : 100 from II in control medium.
V = Inoculation 1 : 100 from II in medium + 0.015% sulfanilamide.

in exactly the same way as if they had remained in the original environment. This excludes transformation of the substance.

C. Three tubes containing 5 ml of 50% blood broth medium are inoculated with the same amount of streptococci *i.e.* one standard drop of a 1 : 1000 diluted 24 hours old culture (tubes I, II, III). To two (II and III) of them sulfanilamide has been added to make a concentration of 0.015%. After three hours to one of the latter (III) is added one drop of a 1 : 100 dilution of the original 24 hours culture of streptococci 1). Of the three tubes data for growth curves are determined. They are presented in Fig. 3.

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1) This culture is at its saturation. So the number herein is the same as it was three hours before.

In the same figure are plotted the curves A (theor.) and B (theor.). They are constructed on the following theoretical base:

A. to the number counted at the third hour in tube II (sulfanilamide 0.015%, no re-inoculation) is added 10 × the initial number of the first inoculum; hereof the logarithm is taken. The further part of the curve is drawn parallel to that of tube II. This would be the curve of tube III if the substance was altered in the period before re-inoculation.

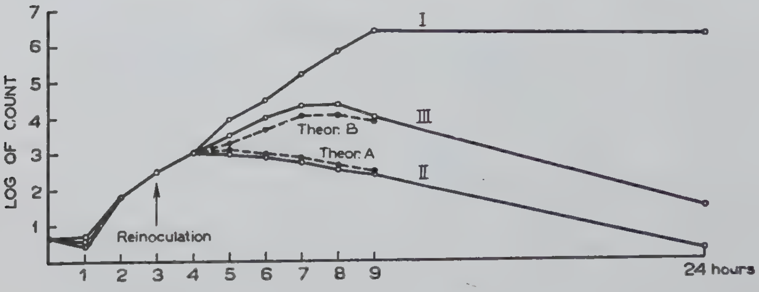

Fig. 3.

I = Control
II = Sulfanilamide 0.015%.
III = II re-inoculated on third hour with 10 × the initial inoculum.
Theor. A = Curve III if substance was transformed.
Theor. B = Curve III if sulfanilamide remains unaltered.

B. to the number counted in tube II are added 10 × the numbers of this same curve in such a way, that to the number determined at the third hour was added 10 × the number of the first inoculum, to that of the fourth hour 10 × the determination of the second hour and so on. The numbers were than logarithmised and plotted in curve B. This would be the curve of tube III, if the re-inoculated cocci had to pass the same lag phase as the initially inoculated cocci, indicating that the drug was unaltered. As Fig. 3 indicates the experimental curve corresponds as well as may be expected with the theoretical curve B.

Summary and conclusions.

The problem of alteration of the sulfanilamide molecule as the *conditio sine qua non* for its action as a germicide is dealt with. Its fundamental aspect is considered. It is demonstrated in two different ways, that in the medium the sulfanilamide need not to be altered for its action. There remains however the possibility that the drug may be transformed in or on the surface of the micro-organism (8). This however is very improbable. For if the cocci are transferred from the sulfanilamide containing medium to a normal one they re-establish immediately their normal growth. This process proves a rapid diffusion of sulfanilamide — altered or unaltered — out of or from the surface of the bacterial cell. Or at the least it is proven, that the active substance, be it the native molecule or not, is not retained by the cocci. In the case of sulfanilamide the osmotic pressure of the drug will maintain a sufficient concentration in the cocci. In the case of transformation of the molecule the osmotic pressure of the new substance outside the cocci however is zero or nearly zero under all conditions. If the substance had to be transformed before action, this might only be realised by a sufficient concentration in the medium. This not being the case is proven in the experiments B and C. It must be admitted that we can not exclude the possibility that the active substance, altered or unaltered sulfanilamide, is consumed, inactivated or the like inside the cocci.

We assume to have proved that any theory for explanation of the mechanism of the sulfanilamide action as to be due to transformation of the molecule into an actual germicide is build on an insolid base. The sulfanilamide as such is the active molecule.

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(From the Laboratory of Hygiene of the University, Utrecht).

ON THE ACTION OF SULFANILAMIDE.

V. THE ACTION ON ANAEROBIC GROWTH [1]

by

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1. INTRODUCTION.

LOCKE, MAIN and MELLON (7) explain the characteristic action of sulfanilamide by an inactivation of bacterial catalase by sulfanilamide, or — as the anticatalase properties of the drug sulfanilamide were minimal — by its oxidation products *e.g.* aminobenzenhydroxamide (cf. (8)). There are three main objections against this theory:

1. no direct action of sulfanilamide on bacterial catalase could be demonstrated (5).

2. a transformation of sulfanilamide into some other active substance outside the cocci was also excluded (6).

3. the bacteriostatic action of sulfanilamide on streptococci is much enhanced by the addition of blood, which is a rich source of catalase.

A further argument in favour or against the theory can be deduced from the action of sulfanilamide on anaerobic growth: anaerobiosis excludes formation of peroxide and sulfanilamide according to the theory should not affect growth under anaerobic conditions. A bacteriostatic action of sulfanilamide on anaerobes was already shown for *Clostridium sporogenes* (10). Also BROH-KAHN (2) demonstrated the action of this drug in nitrate containing anaerobic media. As FOX and JANEWAY (3) pointed out however, in these media the NO_3 takes the role of O_2 and peroxide might be formed.

SHINN, MAIN and MELLON (9) report a reduced activity under low oxygen-tensions. At very low tensions however ($< 0.04\%$), they find sulfanilamide active again. They suppose another mechanism of sulfanilamide action in this case.

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[1] Fourth communication: H. W. JULIUS and K. C. WINKLER, Antonie van Leeuwenhoek **7**, 153, 1941.

As these experiments were of a qualitative character we present in this paper some quantitative experiments about the action of sulfanilamide in anaerobic media.

2. METHODS.

1. For counting viable bacteria the method, described by Julius (4), was used. Usually 5 ml of culture medium — in a small flask with some glassbeads — were inoculated with a standard droplet (28 mg) of a 1 : 1000 dilution of a 24 hours old broth culture of the used strain and incubated whilst a constant stirring by slow rotation on an inclined disc was kept up. Every hour one standard-drop of this culture (diluted if necessary) was added to 2.8 ml of liquefied broth agar (or blood broth agar) in a small flask.

During coagulation the flask was rotated at a speed of about 1000 r.p.m. in a horizontal position. The coagulated agar coats the wall of the flask evenly whilst the bottom remains free. After 24 hours incubation the flask is mounted on a screw-gear and passed under a slit. As the width of the slit is the same as the speed of the screw, no colonies can be counted twice. The passing colonies in the thin layer of agar are counted through a lens, The method is economical, requiring only small quantities of agar and glassware. Every point in the reproduced growth curve represents the average of two independent counts.

2. Growth curves from anaerobic cultures were obtained with the same technic. As the hourly sampling from strictly anaerobic cultures in this case requires complicated apparatus, a number of parallel cultures were used, one of those being opened every hour. The continuity of the curves sufficiently proves the suitability of this method.

Anaerobic conditions were obtained by filling the inoculated medium in Thunberg tubes, and evacuating with a water suction pomp until the fluid had boiled for exactly thirty seconds (two minutes if the medium contained blood). Methylene blue is discoloured after some hours of growth. Leucomethylene blue remains colourless.

3. EXPERIMENTAL.

A. Action of sulfanilamide after reduction of the oxygen tension by nitrogen.

Culture tubes (4 cm diameter) were filled with 8 ml peptone water and 2 ml distilled water or 2 ml of a 0.5% solution of sulfanilamide and inoculated with one drop of a 1 : 1000 dilution of a 24 hours broth culture of *Bacterium coli*.

To one culture tube, containing sulfanilamide and a corresponding control, glassbeads were added and both tubes fixed on an inclined rotating disc for mixing, as usual. Through a second and third set of two tubes sterile air resp. sterile nitrogen was bubbled, relying on the gas current for mixing. Every hour samples were taken and the number of bacteria per drop (28 mg) were counted. The results are collected in Fig. 1.

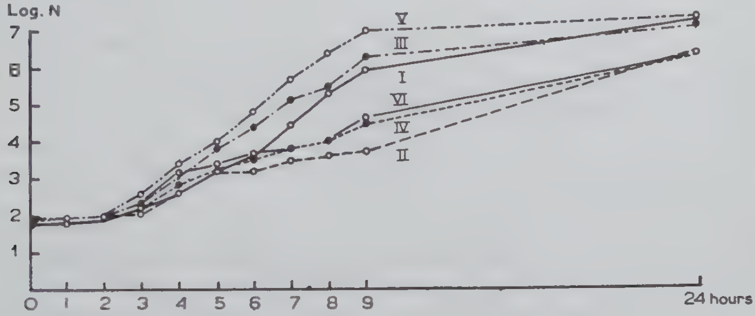

Fig. 1. Influence of oxygen tension on sulfanilamide action. *Bacterium coli* in 1% peptone water.

I = Control } a current of nitrogen was bubbled through
II = 0.1% sulfanilamide } the media.
III = Control
IV = 0.1% sulfanilamide
V = Control aerated
VI = 0.1% sulfanilamide aerated

For the cultures without sulfanilamide the growth is somewhat faster in the order air-control-nitrogen; not much difference is visible however in the sulfanilamide curves. Though the action of sulfanilamide on *B. coli* is not very pronounced in this medium, it is clear however that a reduction of the oxygen tension by nitrogen does not influence the action of sulfanilamide.

With streptococci in 50% blood broth analogous results were obtained.

B. Action of sulfanilamide on anaerobic growth.

1. *Anaerobic conditions maintained by Na-thioglycolate.*

The addition of sulfhydryl compounds to culture media establishes a low oxydo-reduction-potential which permits the developing of anaerobes even in open contact with air (1).

B. coli.

Media: aerobic: 1% peptone, 0.05% agar in distilled water, initial pH = 7; anaerobic: 1% peptone, 0.05% agar, 0.2% Na-thioglycolate in distilled water, initial pH = 7.

The agar is added to decrease O_2 diffusion in the medium. Stirring of the cultures increases the oxydo-reduction-potential by O_2 diffusion. So the inoculated media were divided over ten tubes and incubated. Every hour one tube was opened and the number of viable bacteria counted with the described technic. The four series of tubes contained 4 ml of medium and 1 ml of a 0.5% solution of sulfanilamide in distilled water or 1 ml of distilled water for the two control series. All tubes were inoculated with one drop of a 1 : 1000 dilution of *B. coli*. To every tube one drop of methylene blue 1 : 5000 was added. The anaerobic media remained colourless with a small blue superficial layer during the experiment, thus indicating the anaerobic condition.

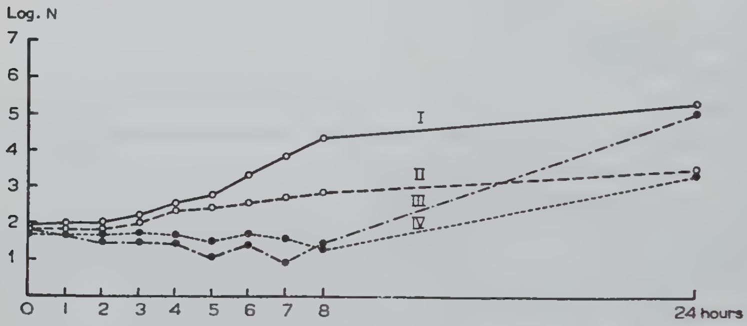

Fig. 2. *B. coli* in 1% peptone water.

I = Control aerobic
II = 0.1% sulfanilamide aerobic
III = Control anaerobic (thioglycolate)
IV = 0.1% sulfanilamide anaerobic (thioglycolate)

The four growth curves are reproduced in Fig. 2. The number of viable bacteria per drop (28 mg) is given on a log scale as ordinate. Though the thioglycolate containing cultures show a prolonged lag time the sulfanilamide action is quite distinct.

Streptococci.

The experiment was carried out in the same way as with *B. coli*. The medium was 50% blood infusion broth with or without 0.015% sulfanilamide. The anaerobic condition was obtained with 1.25% Na-thioglycolate. The growth curves showed much the same features as in the latter experiment with *B. coli* (prolonged lag time).

The action of sulfanilamide in the anaerobic cultures was quite clear. As the method in the next section proved more apt for our subject (no prolonged lag time), further experiments along this line were abandoned.

2. *Anaerobic conditions obtained by evacuation.*

One might argue that thioglycolate has some antibacterial properties itself as is shown by the prolonged lag time. So in the following experiment anaerobic conditions were obtained by evacuating the cultures in Thunberg tubes as described..

B. coli.

40 ml 1% peptone in distilled water and 10 ml distilled water or 10 ml of a 0.5% sulfanilamide solution were inoculated with one drop of a 1 : 100 dilution of a 24 hours broth culture of *B. coli*. Immediately both samples were divided over 5 Thunberg tubes and 5 normal culture tubes. The Thunberg tubes were evacuted and all tubes incubated at 37° C., and shaken hourly. After three, six, nine and twenty four hours one tube of each series was opened, thoroughly mixed and the number of bacteria per drop counted. By adding one drop of sterile methylene blue solution to the fifth tube of each series, the anaerobic condition in the evacuated tubes was proven.

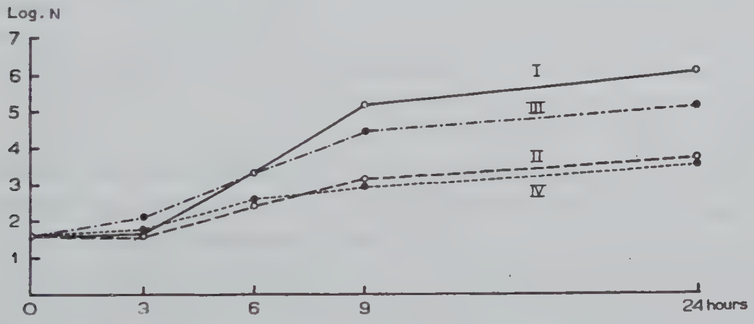

Fig. 3. *B. coli* in 1% peptone water.
I = Control aerobic
II = 0.1% sulfanilamide aerobic
III = Control anaerobic (Thunberg tubes)
IV = 0.1% sulfanilamide anaerobic (Thunberg tubes).

The four growth curves are reproduced in Fig. 3. A distinct action of sulfanilamide in anaerobic conditions is shown. The final level is higher in the aerobic control culture but hardly any difference among the sulfanilamide cultures occurs.

Streptococci.

An analogous experiment with streptococci is reproduced in Fig. 4.

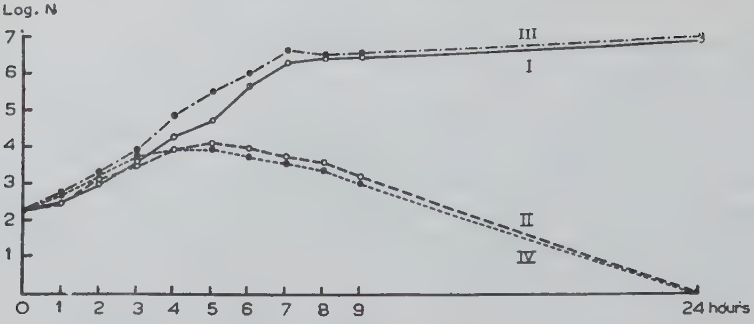

Fig. 4. *Str. haemolyticus* in 50% blood. broth.
I = Control aerobic
II = 0.1% sulfanilamide aerobic
III = Control anaerobic (Thunberg tubes)
IV = 0.1% sulfanilamide anaerobic (Thunberg tubes)

To 60 ml of horse blood and 60 ml infusion broth (pH = 7.6) 4.5 ml of a 0.5% solution of sulfanilamide, resp. 4.5 ml distilled water was added. Both mixtures were inoculated with one drop of a 1 : 10 dilution of a twenty four hours old culture of *Streptococcus haemolyticus* in blood broth. Each mixture was divided over 10 Thunberg tubes and 10 normal culture tubes. The Thunberg tubes were evacuated until two minutes after boiling of the fluid. The blood broth showed the dark red colour of haemoglobine. All tubes were incubated at 37° C., and shaken hourly. One tube of each series was opened every hour and the number of viable bacteria in one drop was counted.

The results are collected in Fig. 4. The growth curves for aerobic and anaerobic cultures are practically identical and the action of sulfanilamide in both cases shows very clearly. The same type of curve was always arrived at in this laboratory in a great number of experiments with aerobic cultures of streptococci containing sulfanilamide (11).

The anaerobic condition of the blood broth might be doubted and though we do not think the objection a very serious one, we can not prove the contrary. So we repeated the experiments with glucose broth.

To 100 ml 1% glucose infusion broth 4 ml of distilled water or of a 0.5% solution of sulfanilamide were added. Both mixtures were inoculated with one drop of a 1 : 20 dilution of a 24 hours culture of *Streptococcus haemolyticus* in blood broth medium. Each mixture was then divided over 10 Thunberg tubes and 10 normal culture tubes. The Thunberg tubes were evacuated for thirty seconds after boiling of the fluid. All tubes were incubated at 37° C., and shaken hourly. One tube of each series was opened every hour and the number of viable bacteria in one droplet (28 mg) was counted.

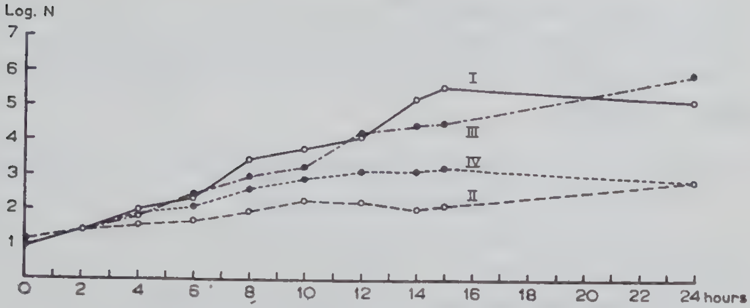

Fig. 5. *Str. haemolyticus* in 1% glucose infusion broth.
I = Control aerobic
II = 0.1% sulfanilamide aerobic
III = Control anaerobic (Thunberg tubes)
IV = 0.1% sulfanilamide anaerobic (Thunberg tubes)

The curves of Fig. 5 show that there is hardly any difference between anaerobic and aerobic cultures in the action of sulfanilamide.

So we can conclude that sulfanilamide acts on *B. coli* and streptococci cultured aerobically or anaerobically in the same way *i.e.* the type of growth curve is nearly identical. The action of sulfanilamide on aerobic and anaerobic growth needs therefore be due to the same mechanism.

The supposition of Shinn, Main and Mellon that the action of sulfanilamide in anaerobic condition would differ from its aerobic action is not very probable in itself. We can bring some evidence which shows this improbability to be such as to rule it out practically:

1. The lag time of sulfanilamide action, so characteristic for this drug, is in either condition visible in the curves.

2. It became evident during these experiments that a variety of conditions (size of inoculum, inclination of the log phase of the growth curve, pH of the medium, presence of blood etc.) influenced aerobic and anaerobic cultures similarly.

3. Woods (12) showed that para-amino benzoic acid was antagonist to sulfanilamide in very low concentrations. Though some other substances counteract sulfanilamide, no other substance was found as yet which equals para-amino benzoic acid. It seems therefore that para-amino benzoic acid re-establishes the specific bacterial mechanism which has been either lamed or affected in another way by sulfanilamide.

The counteraction of para-amino benzoic acid is just as distinct in aerobic as in anaerobic cultures as will be reported on later. It is very unlikely that two different mechanisms of sulfanilamide action should be counteracted quite similarly by the same substance. So we conclude that the mechanism of sulfanilamide action on aerobic and on anaerobic growth is the same.

Summary.

a. The action of sulfanilamide on *B. coli* is not influenced by different oxygen pressures.

b. Sulfanilamide was shown to act on *B. coli* and *Str. haemolyticus* (strain Aronson) cultured anaerobically:

1. by addition of thioglycolate.
2. in evacuated Thunberg tubes.

c. The obtained growth curves with anaerobic cultures show the same characteristics (lag time of action, influence of inoculum, counteraction by para-amino benzoic acid) of sulfanilamide action as the aerobic controls. The mechanism of sulfanilamide action must be the same in both cases.

d. The presented facts are incompatible with the claim of Main, Shinn and Mellon (8) as to the anticatalase activity of sulfanilamide, in anaerobic cultures no hydrogen peroxide being formed.

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SUR LA FERMENTATION DE LA GLUCOSE PAR *CL. TETANI*

par

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(Reçu le 2 septembre 1941).

En 1938 PRÉVOT et KIRCHHEINER (13) ont fait apparaître une publication sur la fermentation de la glucose par *Cl. tetani*. En 1939 elle a été suivie par une communication de la part de BOORSMA, PRÉVOT et VEILLON (3) dans laquelle cette fermentation de la glucose a été étudiée de plus près. Les derniers auteurs ont réussi à faire fermenter dans une anaérobiose stricte en à peu près 11 jours environ 12 g de glucose — probablement contenus dans 1 à 2 litres de milieu de culture — par *Cl. tetani*. Ils en ont dressé un „bilan" où la quantité de glucose fermentée et les quantités des produits de décomposition qui en résultaient ont été comparées.

Il est bien surprenant que les auteurs mentionnés ont réussi à faire fermenter une telle quantité de glucose par *Cl. tetani*, ce qui fut bien contraire à l'opinion générale. Il est vrai que KOLLE et HETSCH admettent dans la septième édition de leur manuel (10) bien connu que *Cl. tetani* serait capable de produire de l'acide carbonique, de l'hydrogène et des carbures d'hydrogène par fermentation de la glucose, mais cette supposition a été déclarée inexacte dans la dernière édition de leur manuel (11).

Déjà en 1902 ACHALME (1) déclarait que la glucose n'était pas fermentée par *Cl. tetani*. KENDALL, DAY et WALKER (9) étaient d'accord avec lui. Dans leurs observations sur l'activité biochimique de *B. botulinus*, *B. sporogenes* et *B. tetani* WAGNER, DOZIER et MEYER (18) ne disent rien sur la fermentation de la glucose par *B. tetani*. ANDERSON (2) pense que *Cl. tetani* serait capable de convertir une quantité restreinte de glucose. Ses conclusions cependant ne sont pas basées sur des dosages de la glucose dans le milieu de culture et par conséquent de peu de valeur. REDDISH et RETTGER (16) constataient que la teneur en glucose du bouillon utilisé après l'ensemencement avec *Cl. tetani* diminuait en 10 jours de 0,727% jusqu'à 0,541%, ce qui revient à une fermentation de 25,6% du sucre additionné. Cependant ni gaz ni acide se seraient produits. COLEMAN et MEYER (7) présument que des traces de glucose étaient fermentées par un type nouveau de *Cl. tetani* (type 8), mais leurs conclusions ne sont pas basées sur des dosages du sucre. TEMPÉ, MANZINI et UHLHORN (17) ne mentionnent aucune fermentation de la glucose dans leur étude sur les qualités morphologiques et biochimiques de *Cl. tetani*. Finalement il faut mentionner en ce rapport une publication de GILLES (8), qui comparait les qualités biochimiques d'une dizaine de souches de *Cl. tetani*, isolées de la crotte. Il se basait exclusivement sur la formation éventuelle de gaz et d'acide dans ses tubes à culture et l'auteur a fini son écrit avec la conclusion suivante: „As regards the saccharolytic reactions, the conclusions arrived at from this study are that *Cl. tetani* does not ferment the carbohydrates. Three of the strains failed to produce acid or gas in any of the testsugars, while seven strains produced minute quantities of gas in the different media. This discloses that *Cl. tetani* in general has slight proteolytic properties with little or no fermentative reaction on the carbohydrates, while variation as already noted by other workers occurs with different strains".

A l'exception de la communication de REDDISH et RETTGER et les investigations françaises mentionnées au début de cet article, les autres publications reviennent à dire que la glucose n'est pas fermentée notablement par *Cl. tetani*.

BOORSMA, PRÉVOT et VEILLON (3) pensaient avoir trouvé de l'alcool méthylique parmi les produits de décomposition de la glucose, chose curieuse, dont aucun fait analogue ne nous est parvenu.

Les faits cités, surtout la formation prétendue de l'alcool methylique, nous ont amenés à répéter l'investigation de BOORSMA, PRÉVOT et VEILLON. Nous avons commencé notre expérimentation avec trois souches de la collection du Rijks Instituut voor de Volksgezondheid à Utrecht, à savoir la souche No 12, provenant du Wellcome Institute à Beckenham, la souche U, provenant également de l'Angleterre et la souche Kopenhagen, provenant du Statens Serum Institut à Copenhague.

Nous ne sommes pas parvenus à faire fermenter même la moindre quantité de glucose par une de ces souches. A cette fin nous avons ensemencé les différentes souches dans des milieux de culture de composition très variée, contenant tous 1,5 à 2% de glucose et que nous avons mis à l'étuve de 35° C. dans une anaérobiose stricte pendant 2 à 11 jours. Ordinairement l'expérience se faisait de telle façon que la teneur en glucose fut dosée avant et après la culture en question. Comme nous l'avons dit, nous avons toujours trouvé cette teneur invariable dans les limites de l'erreur de dosage. Le tableau 1 donne un résumé des expériences exécutées ainsi.

Tableau 1.

No de l'expérience	Composition du milieu de culture	Souches utilisées *)	Durée de culture en jours
1	Eau peptonée à 1% + 0,5% de NaCl; pH = 7,1	12	4
2	Eau peptonée à 1% + 0,5% de NaCl; pH = 6,0	12	2
3	Bouillon de veau + 1% de peptone; pH = 6,4	12	6
4	Bouillon de veau + 1% de peptone + 0,1% de K_2HPO_4 + 0,1% de Na_2HPO_4; pH = 6,9	12	6
5	Bouillon de foie (cf. (12)), obtenu par digestion avec de la pepsine „O.P.G."; pH = 5,8	U, 12, K	11
6	Bouillon de veau + 1% de peptone; pH = 5,8	U, 12, K	6
7	Bouillon de caséine [1]; pH = 7,0	U, 12, K	6
8	Bouillon de foie, traité avec de la levure (cf. (4))	U, 12, K	7
9	Bouillon de foie + 0,002% d'acide nicotinique (cf. (5))	U, 12, K	7
10	Bouillon de foie, obtenu par digestion avec de la pepsine „Witte"	U, 12, K	7
11	Milieu de POPE (14) semi-synthétique. . .	U, 12, K	7
12	Bouillon de foie + 0,1% de thioglycolate de soude + 0,0002% de bleu de méthylène (cf. (6))	U, 12, K	7

*) 12 = la souche No 12.
U = la souche „U".
K = la souche „Kopenhagen".

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[1] Obtenu par digestion tryptique de la caséine.

Nous déterminions journellement la teneur en glucides et le pH du milieu nutritif dans quelques-unes de ces cultures. Nous donnons en exemple le cours de l'expérience 5, faite avec la souche 12, dans le tableau 2 qui suit.

Tableau 2.
Le cours de la teneur en glucose et du pH d'un bouillon de foie, ensemencé avec la souche 12.

No de l'échantillon	Durée de la culture en heures	% de glucose	pH
1	0	1.96	5,30
2	23	1,96	5,83
3	41	1,96	6,31
4	88	1,98	6,84
5	114	1,99	6,83
6	136	1,98	—
7	161	1,96	6,96
8	185	1,96	—
9	208	1,95	—
10	257	1,97	6,98

Les chiffres de ce tableau démontrent que la glucose additionnée au milieu de culture ne fut pas fermentée, tandis que l'accroissement assez considérable du pH révèle des échanges nutritifs évidents. Il faut donc que ceux-ci soient réduits à des échanges des sources azotées, dont les produits, l'acide carbonique et l'ammoniaque, proviennent des peptones et des acides aminés ajoutés au milieu nutritif.

Or, Boorsma, Prévot et Veillon mentionnent l'utilisation de la pepsine „Vaillant" pour préparer le bouillon de foie utilisé dans leurs expériences et d'une souche spéciale de *Cl. tetani*, la souche „L". En principe c'est imaginable que la pepsine mentionnée soit capable d'une décomposition spéciale des albumines de la viande et du foie par laquelle le pouvoir saccharolytique des souches tétaniques différentes aurait été stimulé d'un telle façon qu'il serait question d'une décomposition évidente du sucre. Ce n'est pourtant pas bien vraisemblable.

Tout de même il nous a paru important de répéter notre investigation avec la souche „L", employée par les auteurs mentionnés. Après beaucoup de peine nous avons réussi à obtenir cette souche sous le nom de „Tetanus 132" par l'intermédiaire de M. PRIGGE à Francfort s/Main, à qui nous voulons bien exprimer ici notre reconnaissance.

La souche nous parvenait dans un tube rempli de bouillon et dans un autre tube contenant de la gélatine mi-liquide. Les repiquages des deux tubes ont réussi non sans quelque peine et les souches obtenues furent nommées celles „du bouillon" et „de la gélatine".

D'abord l'examen microscopique de ces souches ne montrait rien d'anormal; des bâtonnets courts, assez épais, restant colorés par la méthode de GRAM, ne pouvant pas être amenés à sporulation. Après quelques jours des sujets longs filiformes, quelquefois longs de 100 μ, qui prenaient mal la coloration d'après GRAM et qui ne sporulaient pas, se développaient à côté des bâtonnets courts, ressemblant aux bacilles tétaniques.

Pour étudier leur pouvoir saccharolytique les deux souches „de la gélatine" et „du bouillon" furent ensemencées dans un bouillon de foie + 1,5% de glucose, pH = 5,8. Après dix jours de culture la teneur en sucre de ce bouillon n'avait pas diminuée.

Puis les deux souches furent ensemencées dans du bouillon de veau + 1% de peptone, pH = 7,0, dont la teneur en glucose était 1,44%. Neuf jours plus tard la teneur en sucre de ce bouillon avait diminué jusqu'à 0,47%, de sorte que 67% à peu près de la glucose ajoutée au début de l'expérience avait été fermentée. La toxine tétanique formée, étudiée aux souris, parut avoir un titre de 1/100.000^{me}, c'est à dire, une injection sous-cutanée de 1 cm^3 d'une dilution de la toxine de 1 sur 100.000 tue une souris de 15 grammes en 4 × 24 heures.

Les deux souches mentionnées ont été repiquées maintes fois dans du bouillon et sous le microscope les cultures montraient toujours à côté de quelques bâtonnets sporulants, ressemblant nettement au bacille tétanique, prenant bien le GRAM, des sujets longs filiformes, qui restaient le plus souvent très mal colorés par cette méthode et qu'on ne pouvait pas amener à sporulation. Toujours la glucose fut fermentée, quelquefois même jusqu'à 100% de la quantité présente dans le milieu nutritif, par les souches „de la gélatine" et „du bouillon" repiquées, et toujours la toxine tétanique fut formée avec un titre d'à peu près 1/100.000^{me}. Cette saccharolyse dépendait fortement du milieu. Si la souche avait été ensemencée dans un bouillon de foie, dont le pH était 5,8—6,0, 30% de la glucose présente seulement fut fermentée, tandis que 90—100% du sucre fut fermenté dans un bouillon de veau + 1% de peptone, auquel on avait ajouté des morceaux de veau avant la stérilisation.

Pendant ces expériences nous commencions à nous douter que cette souche „L" pût contenir à côté des bacilles tétaniques une impureté bactériologique, qui devait être responsable de la fermentation de la glucose.

Pour nous éclaircir sur ce sujet nous avons ensemencé les deux souches mentionnées sur gélose nutritive dans une boîte de Petri et les cultures furent mises en anaérobiose à l'étuve pendant sept jours au moins. Sur la gélose se développaient à une exception près des colonies-mixte, consistant en bactéries longs filiformes, ne prenant presque pas la coloration d'après Gram, à côté de bâtonnets épais, sporulants, qui restaient bien colorés par cette méthode et qui avaient tout à fait l'air des bacilles tétaniques.

L'exception indiquée se trouvait dans une des cultures sur gélose où à côté de colonies-mixte plutôt petites s'étaient développées quelques colonies plus grandes à l'air velouté et en forme d'éventail. Ces colonies consistaient en cultures pures de bacilles tétaniques, bâtonnets courts, épais, prenant la coloration d'après Gram et montrant abondamment la sporulation terminale.

D'une de ces colonies nous avons fait une culture dans du bouillon nutritif et M. S. L. Schouten, à Utrecht, eut l'amabilité d'en tirer quelques spores isolés à l'aide du micromanipulateur. Nous voulons bien lui exprimer ici notre grande reconnaissance. Avec ces spores isolés nous avons réussi à obtenir de la souche du bouillon une dizaine de cultures strictement pures qui ne s'écartaient en rien des cultures de bacilles tétaniques normales.

Ces cultures, provenant d'une cellule unique, furent ensemencées dans des ballons remplis de bouillon de veau + 1% de peptone + 1,5% de glucose + des morceaux de veau. Comme témoins un certain nombre de ces ballons furent ensemencés avec les souches „de la gélatine" et „du bouillon". Tous les ballons furent privés de l'oxygène et portés à 35° C. pendant neuf jours. A l'examen aucun bouillon, ensemencé avec une culture, provenant d'un seul spore, ne montrait un changement de la teneur en glucides! Dans toutes les cultures une toxine tétanique s'était formée avec un titre de 1/100.000^{me}. Dans les cultures des souches „de la gélatine" et „du bouillon" par contre 40—50% de la glucose avait été fermentée. A une reprise de cette expérience la glucose dans la culture des souches, provenant d'une cellule unique, ne fut pas fermentée non plus, tandis que dans des cultures des souches „de la gélatine" et „du bouillon" 99% du sucre présent dans le milieu avait été décomposé.

L'expérience suivante démontre que la toxine formée dans les cultures, provenant d'une cellule unique, était la vraie toxine tétanique. Nous mêlions des quantités de 1 cm^3 de la toxine non diluée et des dilutions au $1/10^{me}$ et $1/100^{me}$ à 1 cm^3 d'un sérum antitétanique avec un titre de 250 unités antitoxiques par cm^3, ou bien avec 1 cm^3 de sérum „normal". L'injection sous-cutanée de doses de 0,5 cm^3 de ces mélanges dans la souris menaient aux résultats donnés dans le tableau 3.

Tableau 3.
La neutralisation de la toxine tétanique formée par les souches, provenant d'une cellule unique.

Toxine	No de la souris	Injecté 0,5 cm^3 du mélange:	1 × 24 heures	2 × 24 heures	3 × 24 heures	4 × 24 heures
toxine No 8 des cultures, provenant d'une cellule unique.	1	1 cm^3 de toxine non diluée + 1 cm^3 de sérum antitétanique	—	—	—	—
	2	1 cm^3 de toxine diluée au $1/10^{me}$ + 1 cm^3 de sérum antitétanique.	—	—	—	—
	3	1 cm^3 de toxine diluée au $1/100^{me}$ + 1 cm^3 de sérum antitétanique	—	—	—	—
	4	1 cm^3 de toxine non diluée + 1 cm^3 de sérum normal .	morte			
	5	1 cm^3 de toxine diluée au $1/10^{me}$ + 1 cm^3 de sérum normal	morte			
	6	1 cm^3 de toxine diluée au $1/100^{me}$ + 1 cm^3 de sérum normal	morte			

Le sérum antitétanique exerce donc un pouvoir nettement défini contre la toxine formée dans la culture examinée, un pouvoir que le sérum normal ne possède pas, de quoi donc la conclusion que cette toxine est réellement la toxine tétanique.

La souche „L", d'après Boorsma, Prévot et Veillon une culture pure de bacilles tétaniques, capables de décomposer une quantité considérable de glucose, se compose donc en effet d'une souche tétanique sans aucune faculté saccharolytique et d'une contamination, qui doit être responsable de la décomposition de la glucose.

Il va sans dire que nous avons essayé d'isoler cette contamination en culture pure. Beaucoup d'essais furent faits, mais tous sans aucun résultat. Nous n'avons pas réussi à cultiver ces bactéries filiformes sur gélose, ni en aérobiose, ni en anaérobiose. M. SCHOUTEN a isolé à l'aide du micromanipulateur un grand nombre de ces bactéries sous conditions strictement anaérobies. L'isolation elle-même ne donnait aucune difficulté, mais aucun des bactéries isolés n'a voulu se multiplier. Finalement nous avons essayé à parvenir au but par la manière suivante. Nous ajoutions un volume égal de sérum antitétanique (900 unités antitoxiques par cm^3) à un certain nombre de tubes, contenant à peu près 8 cm^3 de bouillon de veau + morceaux de veau. Nous avons ensemencé les liquides bien mélangés avec les souches ,,du bouillon'' et ,,de la gélatine'' et avec plusieurs souches pures, isolées par M. SCHOUTEN. Tous les tubes restaient à l'étuve de 35° C. pendant sept jours. Toutes les cultures s'étaient développées et montraient sous le microscope l'aspect décrit préalablement. On peut en tirer la conclusion qu'il est impossible d'empêcher le développement du bacille tétanique par l'addition du sérum antitétanique au bouillon nutritif.

De ce qui précède il résulte que nous n'avons pas réussi à obtenir une culture pure des bactéries en forme de bâtonnets minces et de fils longs. De différentes causes peuvent en être responsables, dont deux seules seront mentionnées ici sans que nous voulions les traîter à fond. Il est possible que ces bactéries longs et filiformes soient tellement sensibles à l'oxygène de l'air, qu'ils sont tués pendant le peu de temps qu'ils sont en contact avec l'oxygène de l'air au moment du repiquage de la surface de la gélose d'abord en anaérobiose, respectivement au moment du passage de la goutte de bouillon pendant l'isolation à l'aide du micromanipulateur. Une autre possibilité peut être trouvée dans une telle vie en commun avec les bacilles tétaniques, qu'il soit incompatible avec leur vie de se développer seuls.

Parce que nous ne sommes pas parvenus à obtenir une culture pure de cette ,,contamination'', il nous a été impossible de déterminer ce microbe. Sans culture pure il est impossible ou bien très difficile de savoir si la formation de l'alcool méthylique, que BOORSMA, PRÉVOT et VEILLON attribuaient à la fermentation de la glucose par les bacilles tétaniques, est due réellement à cette contamination. Aussi nous n'avons pas examiné ce problème de plus près.

Cependant on peut dire avec certitude que les bacilles tétaniques, isolés de la souche française „L" mentionnée plusieurs fois précédemment, ne sont pas responsables de cette fermentation, puisqu'ils ne décomposent pas la glucose en quantités sensibles.

Si nous nous demandons en définitive si la glucose est fermentée par les bacilles tétaniques, nous sommes obligés de constater que ce n'est point le cas. Ceci résulte de nos expériences et est en parfaite concordance avec la plus grande partie des publications d'autres auteurs. Selon toute probabilité les résultats curieux de Boorsma, Prévot et Veillon doivent être attribués à des expériences faites avec un souche bactériologiquement impure.

Finalement on nous permettra de consacrer quelques remarques à une question en rapport avec ce qui a été écrit ci-dessus. A quel point l'addition de glucose au bouillon nutritif est-elle utile à la préparation de la toxine tétanique, ou bien la glucose peut-elle être omise? De prime abord on croirait que la glucose qui n'est pas attaquée par les bacilles tétaniques peut autant bien être omise. Pourtant il est bien connu que la glucose est mentionnée dans presque toutes les prescriptions pour la préparation du bouillon tétanique (15).

Pour nous renseigner sur cette question nous avons fait quelques expériences comparatives. Nous avons ensemencé des ballons contenant le même bouillon nutritif glucosé ou non glucosé avec la-même souche du bacille tétanique.

1. Nous avons ensemencé la souche 12 dans cinq ballons contenant 100 cm^3 de bouillon glucosé à 1,5% et dans le même nombre de ballons contenant ce même bouillon, mais sans glucose. Les ballons étaient portés à une température de 35° C. pendant neuf jours. Les teneurs en toxine, déterminées par dosage titrimétrique aux souris blanches, étaient les suivantes:

   a) glucosé: 1/200.000me; 1/200.000me; 1/150.000me; 1/150.000me; 1/150.000me; en moyen 1/170.000me.

   b) non glucosé: 1/150.000me; 1/150.000me; 1/100.000me; 1/150.000me; 1/40.000me; en moyen 1/118.000me.

2. Nous avons ajouté 1,5% de glucose à six ballons contenant presque 750 cm^3 de bouillon tétanique normal. Un seul ballon n'eut pas de glucose. Tous les ballons furent ensemencés au même moment avec la même culture de la souche 12 et les cultures furent mises à

l'étuve pendant neuf jours. Le résultat de la dosage de la toxine fut le suivant:

a) glucosé: 1/400.000$^{\text{me}}$; 1/400.000$^{\text{me}}$; 1/400.000$^{\text{me}}$; 1/400.000$^{\text{me}}$; 1/200.000$^{\text{me}}$; 1/200.000$^{\text{me}}$

b) non glucosé: 1/200.000$^{\text{me}}$.

3. Nous avons préparé deux ballons avec le bouillon tétanique normal, dont l'un glucosé à 1,5%, l'autre non glucosé. Nous avons ensemencé les deux ballons au même- moment avec la-même culture de la souche „de la gélatine". La teneur en glucose dans le ballon „de la gélatine" diminuait de 1,44% jusqu'à 0,47% (il faut noter, que la souche „de la gélatine" est bactériologiquement impure!). Le résultat des dosages de la toxine fut le suivant:

a) glucosé: 1/100.000$^{\text{me}}$.

b) non glucosé: 1/50.000$^{\text{me}}$.

De ces quelques expériences on peut bien tirer la conclusion, que l'addition de glucose au bouillon tétanique améliore nettement la formation de la toxine. Cependant il est très difficile de concevoir la manière, dont cet effet se produit. On ne saurait certainement pas attribuer l'effet aux produits de décomposition de la glucose, qui pourraient être formés en quantités minimales par une décomposition très petite du sucre additionné. A fin de démontrer ceci, nous avons ensemencé un certain nombre de ballons contenant du bouillon tétanique normal glucosé à 1,5% les uns avec le bacille tétanique, les autres avec une culture mixte de bacilles tétaniques et de bacilles du colon. Comme on sait bien, la glucose est fermentée très facilement par ces derniers. Dans les ballons ensemencés avec la culture mixte il ne se formait pas la moindre toxine.

On peut concevoir une autre explication possible en supposant que la glucose, ajoutée au milieu nutritif, tienne le potentiel d'oxydo-réduction à un niveau déterminé (bas). Bien que certainement ce potentiel a une grande influence sur le développement des bacilles tétaniques et sur la formation de la toxine tétanique, la glucose ne peut pas en être responsable, puisqu'elle ne prend pas sensiblement part aux échanges nutritifs.

D'autres expériences seront donc nécessaires pour résoudre la question posée.

Résumé.

Contrairement aux investigations de Boorsma, Prévot et Veillon, respectivement Prévot et Kirchheiner il n'a pas été possible de faire fermenter la glucose dans de différents milieux nutritifs, ni par trois souches de *Cl. tetani*, ni par une souche bactériologiquement pure, isolée de la souche ,,L'', dont les premiers investigateurs se sont servis.

Il faut donc admettre avec KOLLE et HETSCH et avec beaucoup d'autres investigateurs qu'il est absolument impossible de faire fermenter la glucose par *Cl. tetani.* Aussi les produits gazeux formés, l'acide carbonique et l'ammoniaque, résultent exclusivement de la décomposition des produits azotés, provenant des peptones et des acides aminés additionnés au milieu nutritif.

Il est très probable que les prétendus résultats curieux, qu'ont cru obtenir BOORSMA, PRÉVOT et VEILLON, sont dus à l'usage d'une culture bactériologiquement impure.

La formation de la toxine tétanique est certainement favorisée par l'addition de glucose au bouillon tétanique. Nous sommes incertains sur l'origine de cette influence propice.

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LES MUTILATS DU BACILLE CHARBONNEUX

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Pour obtenir des souches asporogènes du bacille charbonneux des méthodes différentes sont indiquées dans la littérature:

1. la culture à une température indiquée;
2. la culture sur des milieux d'une composition bien définié;
3. la culture sur des milieux anormaux;
4. dans le quatrième cas des souches asporogènes surgissent accidentellement.

Dans mes expériences j'ai employé la méthode de PASTEUR et ses collaborateurs (1), savoir la culture à une température de 42° C. Afin d'être certain que les souches d'origine fussent purement sporogènes, je les ai soumises au procédé suivant:

De chaque souche une suspension a été faite dans quelques cc d'eau physiologique; cette suspension après avoir été placée pendant 15 min. dans un bain-marie de 80° C. (pasteurisation) est ensemencée de la manière habituelle sur des plaques de gélose-de-viande afin d'obtenir des colonies isolées. Ces plaques sont incubées 48 h. à l'étuve à 37° C.

En partant de colonies isolées qui, microscopiquement, montrent une bonne sporulation, de nouveau des suspensions sont faites, puis pasteurisées, ensemencées, etc. Ce procédé a été répété cinq fois, de manière d'être sûr, que le but désiré soit atteint, c. à. d. des souches d'origine purement sporogènes.

Les onze souches, dont je disposais, ont été cultivées d'après PASTEUR à 42° C. sur gélose inclinée, à repiquages quotidiens. De chaque cinquième passage la sporulation a été contrôlée microscopiquement (en goutte pendante, après coloration selon GRAM et après coloration des spores selon ALEX KLEIN). Comme critérium de sporulation on prenait la présence de spores extra-bacillaires et (ou) l'apparition de spores distinctement capsulés. Si les spores étaient absents ou peu perceptibles, une suspension d'une telle culture était pasteurisée, ensemencée sur deux plaques de gélose et incubée pendant 48 h. à 37° C. Si les plaques se trouvaient stériles, la culture était repiquée sur gélose inclinée, incubée pendant 24 h. à 37° C. et ensuite gardée à l'obscurité à la température de laboratoire.

Après quelque temps la non-sporulation fut contrôlée de la même manière. On a pu constater ainsi:

1. que certaines souches ne perdent point leur pouvoir de sporulation;

2. que d'autres souches sont asporogènes pendant quelque temps, pour perdre bientôt cette non-sporulation;

3. qu'il y a quelques souches qui gardent leur asporogénie assez longtemps ou peut-être même pour toujours.

Les souches asporogènes du bacille charbonneux, ainsi obtenues, furent comparées avec leurs souches-mères en étudiant leur morphologie et leurs propriétés culturelles. Pour des détails ultérieurs concernant les résultats de cette comparaison je me permets de référer le lecteur à ma thèse (2), dont ci-dessous un bref résumé.

Les souches asporogènes différent des souches-mères sporulées (outre leur asporogénie):

1. par une diminution de leur virulence pour la souris blanche; parfois elles sont même avirulantes;

2. par une sensibilité augmentée, donc résistance diminuée pour le bactériophage;

3. par la perte de certaines propriétés biochimiques, p. e. l'absence de la forme de ,,sapin renversé'' en piquage de gélatine; la non-coagulation du lait; la perte de la faculté de fermenter le sérum coagulé de Loeffler, etc. Toutes ces propriétés varient d'après les souches.

Il est intéressant d'étudier la durée de l'asporogénie. L'intervalle entre deux repiquages pour le contrôle de l'asporogénie était en général moins qu'un mois, parfois cependant un peu plus long, et, dépendant en quelque mesure de la durée de l'intervalle, des différences se manifestaient dans le sort des souches asporogènes, savoir:

1. Si le delai était moins qu'un mois, les souches asporogènes restaient toutes vivantes, cependant quelques-unes reprenaient leur état sporogène;

2. Si le délai était plus qu'un mois, trois états finals pouvaient se produire:

a. la plupart des souches asporogènes meurent;

b. quelques-unes survivent, tout en perdant leur asporogénie;

c. quelques-unes seulement survivent, tout en gardant leur asporogénie.

(Par contre les souches-mères sporogènes peuvent être gardées durant des mois sans repiquage).

Ceci peut aussi être exprimé en nombres: En partant de onze souches on leur a fait subir cent passages à 42° C., tout en examinant l'asporogénie à chaque 5e passage. Chaque souche donna vingt souches derivées, en total 220 souches-filles. Il parut que 186 de ces 220 souches étaient asporogènes.

Après un seul passage de contrôle à 37° C. la sporogénie était regagnée

par	53 souches
la sporogénie était regagnée plus tard par	52 ,,
perdues par la mort	75 ,,
donc perte de l'asporogénie par	180 souches

Il n'y a que six souches qui ont gardé leur asporogénie jusqu'à présent, savoir:

Abattoir x 40, depuis le 26 juin 1934, nombre de passages de contrôle: 120
Abattoir x 80, depuis le 2 juillet 1934, nombre de passages de contrôle: 114
Bloed x 80, depuis le 8 sept. 1935, nombre de passages de contrôle: 94
Zierikzee x 15, depuis le 21 février 1935, nombre de passages de contrôle: 87
Zierikzee x 65, depuis le 25 juin 1935, nombre de passages de contrôle: 73
Vaccin II x 20, depuis le 26 février 1935, nombre de passages de contrôle: 86

Des manipulations variées ont été effectuées sur ces souches afin de leur rendre leur pouvoir sporogène (culture en bouillon-sang, inoculation à l'animal, etc.), mais sans résultat.

De toutes ces données il suit donc:

1. qu'il est très difficile de se procurer des souches de la bacteridie charbonneux avec asporogénie constante; car même le 70-me passage à 42° C. de la souche ,,Bloed'' qui avait subi, entre août 1934 et janvier 1937, 51 passages de contrôle, et le 85-me passage à 42° C. de la souche ,,Vaccin I'' qui avait subi, entre juin 1935 et avril 1938, 32 passages de contrôle, montraient de nouveau la sporulation, donc respectivement après un délai de 2.5 et 3 ans!

Un autre phénomène intéressant se manifesta au 45-me passage à 42° C. de la souche ,,Wagen'' qui avait subi 72 passages de contrôle entre juillet 1934 et novembre 1939: On injecta une culture asporogène, âgée d'un mois, dans la péritoine de deux souris blanches. De l'une des souris on regagna la souche asporogène, tandis que la souche, cultivée de l'autre souris, parut être devenue sporogène. En outre un repiquage de cette même culture asporogène, âgée de 3 mois, parut être redevenue sporogène, donc au bout de 5.5 ans à peu près!

2. Que les souches-filles asporogènes ne se maintiennent pas facilement dans la nature.

De quelle manière pouvons nous classer le changement de la bacteridie charbonneux, décrit ci-dessus, dans la système de la variation bactérienne?

En étudiant les publications traitant cette variation, deux opinions différentes prévalent:

En premier lieu on peut interpréter ce changement prolongé, respectivement constant, des qualités bactériennes comme un changement de la constitution génotypique du bacille charbonneux. Le bacille asporogène représenterait donc une race nouvelle, une nouvelle espèce.

En ne perdant pas de vue que même des souches du bacille charbonneux, asporogènes depuis longtemps, peuvent retrouver leur pouvoir sporogène, nous serions donc obligés d'accepter en outre des notions comme remutation, inconstance de l'espèce, hérédité de qualités acquises ou perdues, notions qu'on ferait rentrer difficilement dans les opinions génétiques actuelles.

Le second point de vue, avancé par VAN LOGHEM (3), ne se détourne pas de la constance de l'espèce. Pour les microbes même en acceptant théoriquement des changements de la constitution génotypique, il n'en est pas moins vrai que la pratique du changement bactérien ne touche qu'aux réactions pathologiques-physiologiques de l'individu bactérien envers les excitations du milieu, à des changements appartenant donc au phénotype du microbe.

Dans le cas qui nous occupe, l'excitation du milieu consiste dans la culture prolongée à 42° C. La réaction du clone accroissant se manifeste d'abord par l'absence de la formation de spores, perte qui se rétablit immédiatement quand on retourne à 37° C. Au commencement l'asporogénie était donc, selon VAN LOGHEM, une adaptation. Plus l'excitant se prolonge, plus le clone a du mal à se rétablir; l'asporogénie reste constante pendant plusieurs générations de la bactéridie, ce qui nous permet de l'appeler une adaptation raidie ou fixation.

A la fin la régression du clone est telle qu'un retour à la forme précédente semble impossible. En ce cas on peut parler d'atrophie et d'atrophéontes ou encore de mutilats, puisqu'il s'agit ici d'une mutilation constante de la forme.

Comme quelques'uns des clones asporogènes ont regagné le pouvoir de sporulation, même longtemps après la perte de cette propriété (en effet après 2.5 et 5 ans), il n'y a jamais de certitude s'il ne s'agit, chez les clones, asporogènes depuis plus de 7 ans, de fixations ou de mutilation.

Tout ceci ne touche pas à la réalité de la conception de la regression constante. Il y a d'autres exemples du changement bactérien: l'alastrim et le virus de la vaccine sont des atrophéontes du virus de la variole; aussi bien que le bacille de CALMETTE-GUÉRIN, les souches avirulentes de la peste de OTTEN, le bacille paratyphique, qui ne forme pas de gaz, et le colibacille, qui ne fermente pas la lactose, tous sont des atrophéontes de souches-mères normales.

En ce qui concerne le bacille charbonneux, il n'est pas très probable qu'il y aura jamais des clones à changement regressif dans la nature, ces derniers ayant entre autres une résistance diminuée.

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VERZAMELDE GESCHRIFTEN VAN M. W. BEIJERINCK. ZESDE DEEL.

Met registers op alle zes deelen benevens eene beschrijving over zijn werk door G. van Iterson Jr., L. E. den Dooren de Jong en A. J. Kluyver.
Martinus Nijhoff, den Haag, 1940.

Some months ago the sixth Volume of the Collected Papers of M. W. Beijerinck has been published, 20 years after the edition of the initial five volumes. One might expect a posthumous, final volume not to come up in vital interest to the earlier ones; the contrary however is true and it is a match to the others.

Next to some scientific papers published by Beijerinck after he had left the chair at Delft Volume 6 contains three indices (Author Index; Index to organisms; Subject Index), which have been compiled by Dr. H. C. Koning. She has accomplished an extensive task very carefully and the indices are of great help to the reader, who wants to get the most out of the wide variety of subjects masterfully treated by Beijerinck.

In fact Beijerinck used to insert short notes on some subject or other in a treatise on some other matter. So the approach to special items is made hard to the general reader, who will not peruse the whole of the collected papers. During his life many have tried to induce Beijerinck to write a manual on the microbiological technique such as it was thought out and handled by him. He meant to, but never did; probably he was usually too much taken up by the experimental investigation of some subject on hand. In a way the indices may substitute a work of this character and will make his methods easier available.

The chief value of the sixth volume, however, consists in the three treatises in the English language, composed by two of his successors in the chair and by his last pupil and assistant. van Iterson and Kluyver each have treated part of Beijerincks scientific work, the former the botanical investigations, the latter the microbiological ones. The value of these treatises lies in the fact, that they not only have been presented by rarely competent scientists in both their fields, but also that the work of Beijerinck has been discussed in the light of present-day knowledge. And notwithstanding the great increase in knowledge of chemical facts and methods of research, the products of BEIJERINCKS creative imagination can stand this light.

KLUYVER deems BEIJERINCK one of the first great ,,microbiologists'' of his age and probably of all time. In treating his microbiological career he points out the remarkable fact, that many of the most brilliant discoveries in general microbiology have been made in the hardly adequate surroundings of the laboratory of the industrial concern for the production of yeast and alcohol in Delft. The experimental work is treated critically under separate headings in a way that also the less initiated microbiologist is guided to the riches contained in the six volumes. The reader will be greatly impressed by the scientific intuition of BEIJERINCK, who was an initiator in many fields such as virology and ultramicrobiology, which are deemed modern now. Moreover a picture is drawn of the present status of many scientific subjects and an outlook is offered on fields which are in need of further investigation.

The final contribution is made by DEN DOOREN DE JONG, who treats the scientist from the human side. He has composed a biography and has drawn a picture of BEIJERINCK as a scientific worker. The task performed by DEN DOOREN DE JONG was perhaps the hardest. He has succeeded in drawing a portrait very true to life and although a fervent admirer of BEIJERINCK he has been honest enough not to leave out the shade parts. Personalities of the rank of BEIJERINCK may claim for a portrait drawn honestly, not for a portrait by some flattering admirer. In the chapter ,,BEIJERINCK at work'' a very vivid picture is presented of the scientist, of his sparkling vitality and of his characteristic habits and sayings.

Memories treasured by those who have known BEIJERINCK personally will be rendered more vivid; very good up till now unpublished photographs will help others to make a mental picture true to life of the great scientist.

The sixth volume rounds up the impressive work of the five earlier ones very worthily.

M. P. LÖHNIS.

ABSTRACTS

The Editors intend to publish regularly abstracts of microbiological papers written in the Dutch language, which as such may not be easily available to foreign readers. Abstracts of Doctors Theses will be taken up as well. The Editors are glad to have found many willing to collaborate in the composition of these abstracts.

Die Schriftleitung beabsichtigt regelmässig Referate von mikrobiologische Schriften, verfasst in holländischer Sprache und als solche ausländischen Lesern weniger zugänglich aufzunehmen. Auch Doctors Arbeiten werden referiert werden. Die Schriftleitung ist froh viele Mitarbeiter für diesen Abschnitt gefunden zu haben.

La Rédaction se propose de publier régulièrement des comptes-rendus d'articles microbiologiques écrits en langue hollandaise, qui par ce fait peuvent être peu abordables aux lecteurs étrangers. De même des Thèses d'Université seront référées. La Rédaction s'en réjouit d'avoir trouvé beaucoup de collaborateurs pour cette rubrique.

P. H. van Thiel, The use of liquoid (Roche) in the diagnosis of Weil's disease. Nederlandsch Tijdschrift voor Geneeskunde **85**, 3018, 1941.

When the blood is collected in a 1% liquoid solution in normal saline leptospira's not only remain viable for at least 6 days but they will also multiply in the medium. For an immediate diagnosis the method of double centrifuging can be applied to the liquoid-blood mixture, but it gives less satisfactory results than when the blood is collected in sodium-oxalate (Ruys' method). When however the centrifuging process cannot be applied earlier than 3 days after taking the blood, collecting in liquoid is to be prefered. After so long a delay the oxalate plasma contains too many small corpuscles which make the examination of the sediment impossible. In the liquoid-plasma the corpuscles are fewer, especially when it is kept at room temperature.

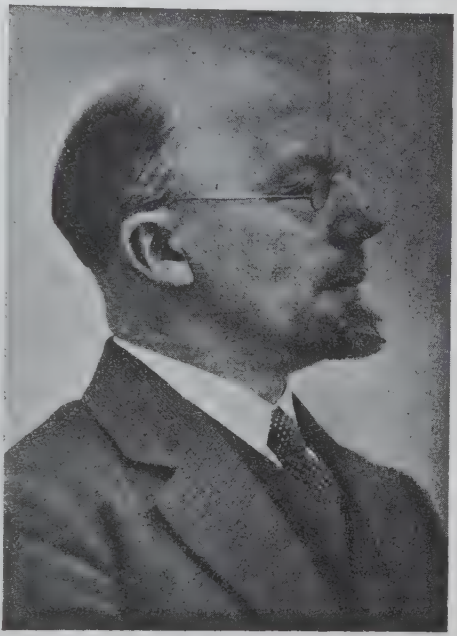
A. Ch. R.

J. J. Th. Vos, Encephalitis toxoplasmotica. Nederlandsch Tijdschrift voor Geneeskunde **85**, 2401, 1941.

The section of a 5 weeks old child, which had suffered from indistinct cerebral symptoms showed a chronic necrotising inflammation in several parts of the brain. Some of the lesions showed signs of calcification, which made it probable, that the disease had set in before birth. At first the cause of the encephalitis was unknown, but after the publication of cases of toxoplasmatic encephalitis by WOLF and COWEN the author re-examined the sections and was then able to demonstrate the toxoplasma in several foci. Some were lying in so called cysts, others free. A similar case has been published by C. C. DE LANGE (Nederlandsch Tijdschrift voor Geneeskunde **85**, 1151, 1941). Here a child 3.5 months old was concerned the disease of which initially was described as a case of hydrocephalus. Later the disease was identified by WOLF and COWEN as a toxoplasma meningo-encephalitis.

A. CH. R.

PROF. DR. J. J. VAN LOGHEM
1916—1941



November 6th 1941 was the 25th anniversary of the inauguration of van Loghem as a Professor at the University of Amsterdam. He entered on his duty by the holding of an address on „Climate and Disease". The editors use this opportunity to offer the readers a complete bibliography of his work. A short biography and a review of his scientific work may precede.

Johannes Jacobus van Loghem was born on April 10th, 1878 at Amsterdam. Here he attended the „gymnasium" (grammar school), and was enlisted as a medical student at the Amsterdam University in 1895. In December 1902 he passed his final medical examination and June 11th 1903 he got his doctor's degree *cum laude* on a thesis: „The Colon and Mesocolon of the Primates". L. Bolk was his promotor. Subsequently he worked with Metschnikoff in the Institut Pasteur in Paris and with the pathologist-anatomist H. Chiari in Prague; in 1905 and 1906 he was assistant of Prof. Ruitinga in the „Binnengasthuis" at Amsterdam. In the following year he was appointed assistant of the professor of Hygiene Saltet and moreover admitted as „Privaat Docent" of Bacteriology. 1908 and 1909 he spent in Netherlands East India as Substitute Director of the Pathological Laboratory at Medan. The year 1911 he was anew in East India as the leader of the fight against plague in Malang. In 1912 he was appointed Director of the Department of Tropical Hygiene of the „Colonial

Institute" at Amsterdam, and in 1916 as a Colonial Institute Professor n Tropical Hygiene at the Amsterdam University. 1919 he spent once more a year in East India. In the same year he was nominated professor extra ordinarius in Tropical Hygiene at the Agricultural University of Wageningen. In 1923 he succeeded his former master SALTET as ordinary professor in Hygiene and Medical Police at the University of Amsterdam.

Among his other scientific tours we mention his sojourn in North Africa in the winter of 1913—1914 and his visit in 1929 to a number of hygienic institutions for the purpose of teaching as well as research in the U.S. in response to an invitation of the Rockefeller Foundation. Again in 1936 VAN LOGHEM payed a short visit to Java and Sumatra.

During the academic year 1939—1940 VAN LOGHEM was Rector Magnificus; from 1938 till August 1941 he was Dean of the Faculty of Medicine.

VAN LOGHEMS years of study have been strongly influenced by HUGO DE VRIES, who in those years of tense scientific life succeeded in the awakening of a sustained interest in genetics also in some medical students. This may explain his tendency to classify from a biological viewpoint the experiences he collected in the bacteriological field in India and the Netherlands and to ponder over the problem of bacterial variability. As a result he banished bacteriological variability from the field of genetics where it was deemed to belong by microbiologists and presented the known data in a new frame: the physiology and pathology of the bacterial individual.

The study of variability had led him moreover to a further analysis of the meaning involved by parasitism. In contradistinction to the conception of KOCH who considers the disease germ as an obligate parasite in contrast to the saprophyte, he leaves more room for the conception of symbiosis in the aetiology of infection. In this way the existence of a border zone formed by commensalism and parasitism was recognised; this recognition would lead subsequently to the new conception of commensal infection (1929).

The study of commensal infection borders directly on the physiology of species. The resistence of different species against the commensal microbes which exert a definite aggressive pressure is a normal property of the species. The lacking or downbreak of this physiological resistence — by subnutrition, disease, disturbance of the heat regulation, old age, wounding, etc. — may lead to commensal infection. Abnormal quantitative relations between the host and the commensal may lead to this as well.

Perusal of the bibliography and the list of Doctorate Thesis composed under the direction of VAN LOGHEM learns that initially the investigation of bacterial variability bore chiefly on the bacteria of the typhus-paratyphus-coli-group, the dysentery group and the proteus group, a study being made of biochemical features as well as of the antigenic structure. Lateron the investigations have extended over diphteria bacteria, vibrios and *Bacillus anthracis.*

In his initial observations of Proteus the complexity of the antigenic structure was met with already (1905); he has taken up this object repeatedly, also with his pupils GROOT (1917), BOELMAN (1923), CASPARÉ (1936) and DE BUSSY (1939). Together with KORTHOF (1918) he demonstrated by means of cultivation in collodion bags in the peritoneal cavity of guinea pigs the variability of the antigenic structure of atoxic Shigella strains; in this way proof was furnished that Flexner and Y-strains belong to a same species. Together with TER POORTEN (1920) and TANG PING IE (1921) he continued his study of the typhus-coli-group. ARNTZENIUS studied Streptococci (1925), BOTMAN the variability of Vibrios (1936), SIEMENS *Corynebacterium diphtheriae* (1938), BEKKER *Bacillus anthracis* (1938).

The study of differences between the members of the typhus-coli-group, which proved the existence of transitions between commensalism and parasitism, has provided the further work of VAN LOGHEM with a stable basis. In a lecture for the ,,Genootschap voor Natuur-, Genees- en Heelkunde" held on January 24th, 1919 on this subject he already claims that: ,,variability might be a bacterial function of a same nature as the manifestation of any other property". Recognising variability as a function he is led to compare the species in this group and notes that along with the increase of the parasitic function variability decreases.

Following this course VAN LOGHEM was led to the investigation of the natural antibodies, the occurrence of which he explains not merely as being due to a physiological ripening, but more especially as caused by a physiological response to the resorption of all kind of saprophytic and commensal antigenic components.

We will draw the attention to two more items in the investigations of the typhus-coli-group. One bears on the clones of a paratyphus B strain on the border line of commensalism and parasitism, which developed colonies of serologically specific character next to non-specific ones. This finding would soon be substantiated by the work of ANDREWES and others, and would lead to the distinction of monophasic and diphasic species of *Salmonella*.

The other item bears on the property of some typhoid clones to comply with ammonia as a source of nitrogen. VAN LOGHEM tends to the view that this is to be considered as atavism in the meaning BEIJERINCK adhered to this term and he classifies in his theory of variability this phenomenon as degeneration. His notions of bacteriological variability, such as they could be deduced from his own work and that of his collaborators were summarized for the first time under the title: Variation of Bacteria considered in relation with the individuality of the bacterial clone. The mere title already reveals that his theory is physiological: variation as a function in contradistinction to the usual conception of genetics. Some years ago (1937) in this periodical he presented again his theory of individuality now based on further data, partly from the work of his pupils. This is published in this periodical under the title „On bacterial variation as a normal and a pathological function", in the Annales de l'Institut Pasteur as a paragraph of his study on „les microbes commensaux". Moreover these papers contain the nomenclature, coined by him for the various physiological and pathological appearances (adaptate, fixate, atropheont, mutilate and degenerant) and the definition of the term „Phaenotypus" from a bacteriological point of view.

He has studied the latter conception as to its suitability for bacteriological research and he arrives at the conclusion that the definition of JOHANNSEN (persönliche Beschaffenheit) falls short in meaning. He proposes the substitution by „totality of adaptations of the individual". This definition is suitable for higher organisms, but is especially well suited for bacteria, because it covers the variations of the bacterial clone (individual line) as well. In one of the general sessions of the Third International Congress for Microbiology held in New York (September 1939) he has addressed the meeting on this subject. The address titled „Genotypical constitution and Appearance", held on January 8th, 1940 on occasion of the Dies in the aula of the University, was chiefly devoted to this subject.

Among the bacteriological investigations of VAN LOGHEM we will still mention the following. *Proteus anindologenes* is a new species described by VAN LOGHEM in 1905, which he noted very frequently with artificially fed infants and with infections of the urinal ducts. This organism is of interest not merely by the complexity and variability of its antigenic structure but also by the agglutinability of definite strains by the serum of scrub-typhus. By means of a systematic investigation together with his collaboratress Dr. I. J. DE BUSSY, VAN LOGHEM has made it probable (1939) that in all strains of Proteus in some measure components also proper to Rickettsia occur.

Several papers, among these from his pupils SNAPPER, LAMPE, BOTMAN, GISPEN and MEYER appeared in the course of time bearing on the difference between *Vibrio comma* and *Vibrio eltor* which in various parts is still deemed as questionable. This subject has proved of a more general bearing because it caused the conception haemodigestion. As far as this bears on the classification of the genus *Vibrio*, unanimity has not yet been arrived at. For a summary of VAN LOGHEMS work on the genus *Vibrio* the reader is referred to the Doctorate theses of GISPEN and of MEYER.

Among other bacteriological investigations we merely mention that VAN LOGHEM was the first to isolate the bacterium of the Shiga-dysentery in Dutch East India (1908) and thus started the study of this disease so important in these regions; moreover he was the initiator of the study of paratyphoid forms in the Netherlands East Indies. He was the first to state the occurrence of plague germs in rats in ships in Amsterdam (1913) and he studied bearers of the cholera vibrio on board of ships coming from Russia (1910).

The conception of commensal infection has been used by VAN LOGHEM as basis for an epidemiological classification. He distinguishes the commensal infection which is sporadic and endemic in nature from the parasitic infection which is epidemic and eradicable.

In some publications among them of his pupils KULSDOM, MULLER, MEYER, this epidemiological classification has been made use of in the study of refrigeration diseases, tuberculosis, meningococcosis and Tor enteritis. In his Text book ,,General Hygiene" it has been fully described.

The conception that higher organisms during the whole of their life keep up a continuous struggle against a large number of commensals and parasites is largely connected with the interest VAN LOGHEM takes in natural antibodies. These make out the subject of the Doctorate Thesis of REURINK.

The epidemiological study of plague and yellow fever led van Loghem to some zoological investigations bearing on the biology of rats, flees and mosquitoes. In the latter research Mrs. van Loghem took a part. The systematic investigation of rats in Dutch East India and their parasites was started by van Loghem in a period (1908—10) when merely a few cases of plague some years earlier had been noted and the question was still open whether Netherlands East India might become subject to an epidemic of plague. As a result of this investigation, in view of which van Loghem had also studied the plague problem in British India on occasion of the congress of Bombay (1909), a sharp distinction was made between *Mus rattus*, domestic rat, and *Mus rattus*, field rat; next to biological properties new morphological characteristics could be observed. When some years later plague actually broke out in Java and van Loghem had been charged with the leading of the fight against it in Malang, this investigation supplied directly a rational basis for measures to be taken. Up till now the East India Government maintains the viewpoint that the system of improvement of the houses of the indigenes such as it had been conceived by the health service in agreement with the investigations of van Loghem is the only rational measure in the combat against plague. The application of Otten's vaccin on a large scale is merely a palliative measure to be taken in regions where improvement of the houses for technical and financial reasons lags behind the occurrence of plague.

In this connection the investigation of the plague problem for Europe may be mentioned. By regular biological control of the rats in the ships entering the Amsterdam harbour he could demonstrate that the Netherlands may actually be threatened by plague. In view of this the composition of the population of rats in the Netherlands was studied and more especially the occurrence of *Mus rattus* in Amsterdam and in the rural districts in the provinces of Groningen, Gelderland and Drenthe.

Along with this historical research has been carried out; van Loghem was the first to prove the occurrence of a high mortality in rats on occasion of the historical plague. Moreover by means of data from archives some epidemics of plague in the 17th century in Amsterdam have been mapped out (Doctorate Thesis Dijkstra).

All these data substantiate the conception that the historical bubonic plague in Europe had depended on the plague of domestic rats.

Another exotic disease, the epidemiology of which van Loghem was the first to study in relation to Dutch East India, is yellow fever.

After his attention had been drawn in 1908 to the frequent occurrence of the mosquito of the yellow fever on the East coast of Sumatra, the biology and frequency of this mosquito in Dutch East India have been studied by him together with Mrs. VAN LOGHEM (only later — when monkeys were known to be suitable for experiments on transmission — the problem of yellow fever in Dutch East India would be open to experimental research).

In the epidemiological field of cholera some investigations have been carried out. By means of a systematical examination of hundreds of sailors it was demonstrated that during the reign of an epidemic of cholera in Russia, clinically unrecognised cholera patients could freely enter the harbour of Amsterdam, a fact which threw new light on the quarantine problem in the Netherlands.

In connection with this review it may be mentioned that his bacteriological as well as his epidemiological conceptions have brought him to the taking up of several technical problems. VAN LOGHEM introduced by personal research the BECCARI method for fermentation of domestic garbage in this country; together with his pupils he carried out investigations of the microbiological processes in septic tanks (Doctorate Thesis of SCHUT). He extended his study of commensal infection over refrigeration diseases and hibernal mortality. As a result of a fruitful cooperation with the Institute for Engineers the latter work of VAN LOGHEM provides a medical basis for the work of the commission for study of the regulation of the inner climate of buildings, of which he is a member. By his putting to the test of a natural system of ventilation by means of deflectors VAN LOGHEM has entered the field of hygienical technique. Not merely did he have the class room of the Laboratory for Hygiene ventilated according to his views, but the architects of the new building of the Rijksverzekeringsbank in Amsterdam applied the „VAN LOGHEM" windows.

We will not end up this review without mentioning his deep interest in the social aspects of hygiene and medical science as well as in the teaching of them.

In the period he spent in India, which appears to us as decisive for his microbiological and epidemiological development, his scientific attention was directed to the hygienical organisation of communities. The interest in communities such as have been designed for the first time by him as „compulsory communities" kept hold of him; his wish to visit once again Dutch East India which has been fulfilled in 1936, was more especially due to his interest in the gradual development of the model kampong from the labourerspondok in Deli.

The direct aim of his tour in North Africa in the winter 1913—14 lay in the study of the hygienic organisation of subtropical cities.

Since 1920 van Loghem has advocated warmly the districtal organisation of health services. In connection with designs and wishes in this field he organised (in 1926—27) with his collaborator Dr A. Charlotte Ruys courses for the training of hygienists according to a program which had been drafted by him as a member of the Health Council and approved by the Government. In the thesis of his pupil Groeneveld, who under his direction perused the *Indicia Sanitatis* of Nijmegen — this subject belonging to the program of the union of Directors of Health Institutions in Geneva, of which van Loghem was a member — the conception of districtal organisation of health service is defended making use of data, collected in the Netherlands.

Moreover his contribution to the Nederlandsch Tijdschrift voor Geneeskunde, in which periodical he edits the rubric ,,Arts en Samenleving'' shows his interest in any scientific endeavour which may prove of fundamental significance for the study of public health.

Van Loghems collaboration with the academic teaching in Amsterdam dates as far back as 1907 when as ,,privaat docent'' he organised the practical training in bacteriology in the laboratory of Saltet on a new basis. The present Laboratory for Hygiene with a Department for Tropical Hygiene designed in 1912 by Saltet and van Loghem, and opened in 1917, satisfies up till now high demands. Unremittingly van Loghem has applied himself to keep his theoretical as well as practical teaching up-to-date.

By enlisting Dr Jan Smit in 1923 as a collaborator van Loghem has succeeded in organising the teaching of the general and applied microbiology, as designed for chemists and pharmaceuts of the University of Amsterdam. After the nomination of Dr Smit as professor in Wageningen Dr Kingma Boltjes succeeded him as Lector.

In his teaching of hygiene van Loghem has made abundant use of the wide regions in the epidemiological, immunological, technical, organisatory and genetical fields which he has explored. In his text book ,,Algemeene Gezondheidsleer'' (1935), a new edition of which has recently appeared, this is described.

Among the distinctions, which fell to his part we merely mention the Officers cross of the Order of Oranje-Nassau, which was designed to him after his work during the plague epidemic in Java in 1912 and the Knights cross of the Nederlandsche Leeuw which he received, when he resigned as a Director of the Department of Tropical Hygiene of the Colonial Institute in 1923.

Amsterdam, October 23, 1941. A. CHARLOTTE RUYS.

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Amsterdam, October 23, 1941. A. Charlotte Ruys.

(From the Bacteriological Laboratory, Medical University
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A TROPIN-REACTION FOR THE DIAGNOSTIC OF BRUCELLOSIS

by

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(Received October 20, 1941).

HUDDLESON et al. (3), in 1932, described an ,,opsono-cytophagic-reaction'' which they claimed to be suitable for the laboratory-diagnostic of brucellosis. It was based on the experience that addition of sodium-citrate in a definite amount to the whole-blood, eliminates a great deal of non-specific phagocytosis, whereas specific opsonic action remains unchanged.

This test has only been applied in practice in U.S.A. Unfortunately several investigators were unable to confirm the reliability of HUDDLESON's reaction, especially with regard to the specificity of its results, nor did I acquire data, which were satisfactory enough to recommend the test for routine practice.

Studying a large material I was able to establish the specificity of phagocytosis in the bloodserum of men or animals, suffering from brucella-infection, after the sera had lost their complement by heating.

Here upon I have based a serum test, which I called ,,tropin-reaction'' the description of which first has been published in 1940 (2). In 1941 JERSILD (4) published a similar method, depending upon the same principle, but applying a different technique. His results in brucellosis of man are claimed to be very satisfactory.

The technique of the tropin-reaction, such as it is used by me, is the following:

Sera to be tested are inactivated by heating during 30 minutes at 56° C.

The phagocyting leucocytes may originate either from man or from animals. Five ccm blood collected in glass vials containing 0.2 ccm of a 20 percent solution of sodium-citrate (in physiological salt solution) are centrifugated and the sediment is washed once with physiological water. After decanting carefully, the red and white cells in the sediment are mixed by shaking gently.

A brucella-suspension is prepared by washing off a 24 or 48 hours old agar slant culture of smooth strains of brucella Bang with physiological water, after the condensation-water has been removed by means of a pipette-Pasteur. The turbidity of the suspension is brought up to such a degree that a platin loop may just be visible 2 mm below the surface.

Clean sterile Widal-tubes are provided with: 0.1 ccm of the heated bloodserum which is to be tested, 0.1 ccm of the washed bloodcell mixture and 0.2 ccm of the bacterial suspension. After the contents of each tube have been mixed together, the vials are placed in a waterbath for 30 minutes at 37° C. Then the sedimentated cells are shaken up into the supernatent fluid. By means of a small pipette a droplet is placed at one end of a thoroughly cleaned glass slide and with another polished slide the drop is spread out over the surface of the former slide. Then the film is rapidly dried on a plate, heated to about 40° C., fixed by methyl alcohol, and covered with a GIEMSA-solution (30 drops of GIEMSA-stain in 10 ccm distilled water).

At the end of 20 minutes the spread is washed free from stain and differentiated by bringing 4—5 drops of 1 percent acetic acid into the washing water, which was left on it. After 5—10 seconds the fluid is poured off from the slide and the latter is gently washed with clean water. The colour of the dry film must be more reddish than purple and without any hint of blue.

In stead of colouring the spread with GIEMSA-stain an easier method may be applied consisting of staining with a mixture of methylene blue and carbol fuchsin (1 ccm saturated alcoholic methylene blue-solution, .3 ccm carbol fuchsin and 100 ccm distilled water, PICK-JACOBSON's stain). After 3 minutes the film is washed free from stain and dried. The results of this staining method is satisfactory, although the differentiation of the bloodcells is inferior to that which follows the GIEMSA-stain.

A total of 10—20 neutrophylic polynuclear leucocytes is studied with oil immersion. As index for the determination of the strength of the reaction the number of leucocytes out of 10, which contain more than 5 bacteria is noted.

For a closer study of the details of the technique of the tropin-test several investigations have been carried out, leading to the following conclusions:

Sera from man, cattle, horse and goat suffering from brucellosis prove to afford the best results when using a suspension of living brucella-organisms, though suitable reactions can be obtained as well with cultures killed by heating (56° C.) or by phenol (0.5 percent), even when these suspensions have been stored in the icebox for a considerable time.

Moreover the sera to be tested may be stored in the icebox for several weeks or months without losing their tropin-activity.

The leucocytes may origin from man or from animals, such as cattle, horse and goat. Less suitable is the blood of rabbits and guinea pigs.

The microscopical picture will be clearer when the occurrence of heterophylic agglutinins and the actual bloodgroups are taken into account. As the sera have been inactivated before, hemolysis does not occur.

If the suspension of bloodcells cannot be used immediately after punction, the blood has to be stored in the icebox. Within the first 24 hours practically no decrease of phagocytic activity of the leucocytes can be noted.

After the leucocytes, serum and bacteria have been mixed together, phagocytosis occurs very rapidly. In most cases maximal phagocytosis has been brought about within less than 20 minutes.

Though a few leucocytes seem to take up brucella at room temperature as well as at 37° C., it is evident that the tropin-action is strongly stimulated by keeping the vials at higher temperature (37° C.).

Preliminary investigations made it clear that in rabbits a strong tropin-reaction appeared in less than 12 days after the infection with brucella-organisms had taken place. Using goats, the occurrence of tropins in the blood could be studied more in detail. Complement-fixing antibodies and agglutinins were to be found in the blood serum of a goat as early as 3 days after its infection with a brucella culture and in another goat 4 days after injection of a brucella suspension which had been killed of. Specific tropins appeared in the blood of the former animal, 6 days after its inoculation, in the other one on the 5-th day, the unspecific opsonins having increased already at an earlier stage. The tropin-reaction still gave positive results 14 months after the goats had been treated, the strongest reactions occurring in the animal, which had been inoculated with living bacteria.

The diagnostic value of the tropin-reaction as a routin method has been tested out in spontaneous brucellosis of man, cattle and horse.

Man.

In 76 unselected sera of humans suffering from several diseases, but not from brucellosis, all of which were reacting negatively with the agglutination test (1 : 50 and more) as well as with the complement-fixation test (0.2 ccm and less), no positive tropin-reactions have been noted. When, however, unheated sera were used, 47 out of 76 sera seemed to possess an opsonic action.

A special group is composed out of 6 veterinary surgeons who although on the moment of the test in perfect health still gave strong positive tropin-reactions (cf. table 1).

Table 1.

Veterinary-surgeons.

No.	Agglutination ¹)	Complement-fixation ²)	Tropin-reaction ³)
1	50 (weak)	0.05	10
2	100	0.2	8
3	100	0	9
4	100	0.05	10
5	200	0.05	9
6	400	0.01	10

¹) Agglutination-titres: 1/50, 1/100, 1/200 etc.

²) Complement-fixation with 0.2, 0.05, 0.01 and 0.005 ccm serum.

³) Tropin-reaction, the index indicating the number of leucocytes out of a total of 10, which have phagocyted 5 or more bacteria.

The positive serum reactions in healthy veterinarians can easily be explained. Since the investigations of Axel Thomsen we know that nearly all individuals who, in their profession, are exposed to infection by brucella Bang, contain specific antibodies towards this micro-organism. In a study of professional diseases of veterinarians in Holland which are transmitted from animals (1939), I have substantiated this finding of Thomsen with regard to complement-fixation. As it is shown in table 1 this applies as well for the tropins. The specificity of these substances in the above cases is rendered very probable by the following observations. The sera indicated by the numbers 2 and 4 (table 1), had also been tested half a year earlier, when both persons still were students and at that moment no antibodies appeared to occur (agglutination, complement-fixation, tropin-reaction). One of them became an assistant in the Obstetrical Clinics of the Veterinary Faculty and the other had settled in a district where infectious abortion was endemic. Both of them now showed strongly positive reactions.

All 11 sera deriving from persons suffering from brucellosis gave strong positive tropin-reaction as well as positive agglutination- and fixation tests. Out of the blood of 5 of these individuals brucella Bang has been cultivated (cf. table 2).

Table 2.

Individuals suffering from brucella infection.

No.	Agglutination	Complement-fixation	Tropin-reaction
1	12.800	0.005	7
2	3.200	0.005	10
3	3.200	0.05	10
4	12.800	0.005	10
5	800	0.005	10
6	400	0.01	10
7	1.600	0.05	10
8	6.400	0.005	10
9	3.200	0.005	9
10	400	0.01	10
11	25.600	0.005	8

Cattle.

In view of the great extension of infectious abortion in the Netherlands, the cattle may be considered to be very intensively exposed to brucella infection. However, in a same way as it is known for highly exposed human beings, infection of cows is not necessarily followed by clinical symptoms of disease.

Out of 72 control sera of cattle, containing neither agglutinins (in dilution 1/25 or more) or complement-fixing antibodies, not one proved to possess brucella tropins. When tested fresh (unheated) 22 of these 72 sera gave a non-specific opsonin reaction.

Most (47) of the 69 sera with an agglutination titre of 1/50 or more or with a positive complement-fixation test derived either from cows which had earlier aborted or from animals belonging to an infected herd (cf. table 3).

Table 3.

69 sera of cattle containing tropins 47, of these cattle suffering from infectious abortion or belonging to an infected herd.

Number of sera	Agglutination-titre	Complement-fixation	Tropin-reaction
8	6400	0.005	10
7	3200	0.005	10
7	1600	0.005	10
2	1600	0.01	10
1	1600	0.05	10
7	800	0.005	10
2	800	0.01	10
2	800	0.05	10
1	800	0.05	8
3	400	0.005	10
1	400	0.005	9
3	400	0.01	10
1	400	0.05	10
1	400	0.2	10
6	200	0.005	10
2	200	0.01	10
2	200	0.05	10
1	200	0.01	9
1	200	0.2	7
2	100	0.005	10
1	100	0.01	10
1	100	0.2 weak	9
1	100	0.01	8
1	50	0.2	10
1	50	0	9
1	25	0.05	9
1	25	0.2	10
1	25	0.2	10
1	25	0.05	6
69			

As appears from table 3 there are several cases where the titres of agglutination- and complement-fixation tests were not conclusive. Here strong positive tropin-reactions appeared of considerable value to support the serological diagnosis.

Horse.

In 58 out of 79 inactivated horse serums no phagocytic action was to be detected, whilst neither agglutinins nor complement-fixing antibodies were present in 47 of these 58. No clinical data of brucellosis were to be obtained from these 58 horses. Five sera agglutinated brucella to a dilution of 1/50, 3 of these also possessing slight complement-fixation activity (0.2).

In one horse serum no tropins were present, whilst the agglutination titre reached 1/100 and there was a strong positive complement-fixation reaction (0.005). This serum derived from an animal, which had formerly suffered from fistulous withers (Bang's disease), which some 4 months prior to the serum investigation had been totally extirpated.

At the other hand I examined the serum of a farmer's horse suffering from brucellosis (fever, increasing thinness, stiffness of the neck, etc.) giving strong positive reactions (aggl. 1/3200, compl. fixation 0.01, tropin-reaction 10), which 11 months after its clinical recovery was fully deprived of agglutinins and amboceptors but still gave a very strong tropin-reaction (index 10).

Table 4 shows the results of the serum investigations of the remaining 19 horse sera with positive tropin-reactions.

Table 4.

Serums of horses with positive tropin-reaction.

Agglutination-titre	Complement-fixation	Tropin-reaction	Clinical data
3200	0.005	10	Extensive withers; brucella cultivated from pus.
200	0.05	10	Fistulous withers; brucella cultivated from pus.
400	0.01	10	Cattle suffers from infectious abortion. Horse: symptoms of Bang's disease. Five days after the serum investigation, spontaneous swelling of the withers, from the contents of which brucella is cultivated.
3200	0.005	10	Fistulous absces of the sternum. Brucella Bang cultivated from the pus.
400	0.005	10	Chronic fistulous absces of the sternum.

Agglutination-titre	Complement-fixation	Tropin-reaction	Clinical data
3200	0.01	10	Undulant fever; increasing thinness; stiff neck.
800	—	10	Fever; general weakness; stiff neck.
3200	0.05	10	Slowness since several weeks; muffishness; increasing thinness; repeatedly lameness and fever.
400	0.005	10	Continual intermittent fever; general weakness.
3200	0.005	10	General emaciation; intermittent fever.
800	0.01	10	
200	0.01	10	
200	0.05	10	
100	0.05	10	
50	0.2	10	
25	0.2	8	
50	0.2 weak	4	
0	0.05	3	
25	0.2 weak	3	

Ten out of these 19 sera derived from horses, which were clinically suffering from brucellosis. From the pus of 4 of them brucella has been cultivated.

In the other 9 cases no direct features of infection with brucella organisms were present. Eight of these were farmer's horses, one was a sledge-horse.

As in nearly all of these cases the agglutination- and complement-fixation reactions gave positive results as well, it is very probable that the existence of the tropins may have been due to former infections, which were healed or to its occurrence in a latent stage without clinical symptoms ¹).

Although in those cases the positive tropin-reaction, as well as the agglutination- and complement-fixation reactions must be considered

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¹) This supposition is supported by the earlier mentioned observations in experimentally infected goats, where antibodies appeared after the injection of brucella and could be detected during a very long period, whilst the animals were in perfect health.

to be of specific nature, they still may entail mistakes in clinical conclusions.

Summary.

Description of a serum test, called „tropin-reaction" for the routine diagnosis of brucellosis in man and animals.

It is based upon the heat-stability of specific tropins, contrary to the heat-sensibility of the unspecific opsonins.

With due regard to latent infections, especially in the case of highly exposed individuals (occupational infections) and in horses, the tropin-reaction is a simple, specific and extremely sensitive diagnostic method, which may prove a valuable addition to the agglutination- and complement-fixation reactions.

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(Aus dem Laboratorium für Hygiene, Bakteriologie und Tropische Hygiene der Reichsuniversität, Leiden).

BIOLOGISCHE METHODEN ZUM ISOLIEREN VON *LEPTOSPIRA ICTEROHAEMORRHAGIAE* AUS WASSER[1]

von

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(Eingegangen am 12 Oktober 1941).

Die biologische Methoden zum Isolieren von *Leptospira icterohaemorrhagiae* aus Wasser, deren Resultate in der Arbeit von einem unser (VAN THIEL (4)) kurz mitgeteilt wurden, werden hier näher erörtert werden.

a. Die Zentrifugalmethode.

In einer Zentrifuge vom Sharples-Typus der Firma Padberg werden 5 Liter Wasser mit einer Geschwindigkeit von 35000 Umdrehungen pro Minute zentrifugiert. Das Zentrifugat wird quantitativ in 25 ccm Wasser gesammelt; die auf diese Weise erhaltene schlammige Flüssigkeit wird während 1.5 Stunden mit der rasierten und skarifizierten Bauchhaut von 2 Meerschweinchen in Berührung gebracht. Dazu wird mit Hilfe von Wachs-Paraffin und Elemiharz ein Glaszylinder derartig auf den Bauch des auf einem Brett aufgespannten Tieres angebracht, dass die in den Zylinder gebrachte Flüssigkeit nicht herausfliessen kann.

Bevor es möglich war diese Experimente zu machen, war es erst nötig sich mit der Zentrifuge vertraut zu machen.

An der Basis der Zentrifuge (Abbildung 1) findet sich eine Spritze mit einer Oeffnung, durch welche das zu untersuchende Wasser in den Zentrifugezylinder hineinspritzt. Es lassen sich drei Spritzen mit Oeffnungen von verschiedener Grösse (weit, mittelweit und eng) dazu verwenden.

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[1] Nach einem Vortrag im Niederländischen Verein für Tropenmedizin am 10 November 1940. Eine kurze Zusammenfassung wurde in den Acta Brevia Neerlandica (vergl. (5)) publiziert.

[2] Während der Mobilisierung als Offizier für Spezialdienste des Militär-pharmazeutischen Dienstes am obigen Laboratorium detachiert zum Studium von militärhygienischen Weilfragen.

Mittels eines Hahnes ist es möglich die Höhe des spritzenden Wassers zu regulieren.

Sobald alles Wasser zentrifugiert ist, wird der Zentrifugemotor abgestellt, und also die Zentrifugierung beendet, sodass keine Zentrifugationsflüssigkeit mehr aus der Ausströmungstülle herauströpfelt. Weil im Zentrifugezylinder die letzten 250 ccm Wasser zurückbleiben, welche abfliessen, sobald der Zylinder zum Stillstand gebracht ist, ist die Zentrifugierung im letzten Versuch noch während 15 Minuten fortgesetzt, nachdem die Flüssigkeit aufgehört hat aus der Tülle herauszutröpfeln.

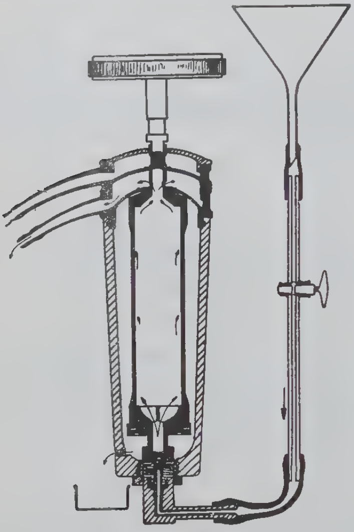

Fig. 1.

Es ist uns leider nicht möglich gewesen in einer solchen Weise zu zentrifugieren, dass kein Wasser verloren geht. Dieses ist nur in solchen Experimenten störend aufgetreten, in denen es sich um Berechnung der erreichte Konzentration handelte. Darum ist die Quantität dieses ,,Leckwassers'' auch nur in diesen Versuchen angegeben worden.

In der Tabelle I findet man diese orientierenden Untersuchungen. An 5 L. Grachtenwasser wurden etwa 44 ccm Kulturflüssigkeit von *Leptospira biflexa* (*pseudoicterogenes*) hinzugefügt, sodass sich etwa 40 Leptospiren in 10 mikroskopischen Gesichtsfeldern (mit starkem Trockensystem) vorfanden. Aus der Anzahl, die festgestellt wurde nachdem das schlammige Zentrifugat aus dem Zylinder in 25 ccm Wasser gesammelt worden war (dies soll ganz genau und vorsichtig mit einer beim Apparat gehörenden scharfen Messersorte und wenigstens 5 Ausspülungen von sterilem Leitungswasser gemacht werden), liessen sich die Leptospirenverlust und die endgültige Leptospirenkonzentration berechnen.

Es hat sich gezeigt, dass das Zentrifugieren am besten gelingt (keine Leptospirenverlust und 250 malige Konzentration), wenn die engste Spritzöffnung verwendet wird und das Wasser bis zur Höhe von 10 cm in den Zylinder hineinspritzt. Der Motor muss abgestellt werden, sobald keine Zentrifugationsflüssigkeit mehr aus der Tülle herauströpfelt. Wenn man dann die letzten 250 ccm Wasser aus dem Zylinder auffängt, stellt es sich heraus, dass die Konzentration der Leptospirenzahl durchschnittlich zweimal die der Untersuchungsflüssigkeit beträgt. Wenn die Zentrifugierung noch während 15 Minuten nach Aufhören der Herauströpfelung der Zentrifugationsflüssigkeit fortgesetzt

Tabelle I.

Besonderkeiten betreffs Spritzöffnung und Spritzhöhe des Wassers	Ende der Zentrifugierung	Dauer der Zentrifugierung in Stunden	Leckwasser (ausser Zylinderinhalt)	Leptospirenverlust	Endgültige Leptospirenkonzentration beim Suspendieren vom Zentrifugat (aus 5 L. Wasser) in 25 ccm Wasser
mittelweit	Ende Auströpfelung Zentrifugationsflüssigkeit	?	300	90 %	20 Mal
eng; 1 dm	,,	1½	250	35 %	120 Mal
eng; 2 dm	,,	1	500	59 %	84 Mal
eng; 2 dm	,,	½	150	52 %	96 Mal
eng; 1 dm	,,	1½	100	kein	250 Mal
eng; 1 dm	,,	1½	150	kein	191 Mal
eng; 1 dm	15 Minuten nach Auströpfelung Zentrifugationsflüssigkeit	1½	100	kein	235 Mal

wird, beträgt die Konzentration 5.5. Es ist also deutlich, dass es schädlich ist länger zu zentrifugieren als die Zeitdauer, während die Flüssigkeit aus der Tülle tröpfelt. Dass in den letzten drei Versuchen (Tabelle I) keine Leptospiren, ungeachtet des Verlustes von Leptospiren aus dem letzten Zylinderinhalt und aus dem „Leckwasser", verloren gegangen sind, wird durch die Vermehrung der Leptospiren hervorgerufen. Während der Zentrifugierung steigt nämlich die Temperatur des Wassers im Zylinder um ungefähr 7°C. Allenfalls konnten die Leptospiren erst ungefähr eine Stunde nach Ablauf der 1.5 Stunden dauernden Zentrifugierung gezählt werden.

Auch experimentell konnte eine Leptospirenvermehrung während der genannten Zeit nachgewiesen werden. Man erhitzt 2 Liter saprophytische Leptospiren enthaltendes Wasser während einer halben Stunde auf 27°C. und lässt es dann während 2.5 Stunden bis auf 20°C. abkühlen; dann beträgt die Leptospirenzahl in einem Wassertropfen vor dem Versuch 11, nach Ablauf der Abkühlung 14 Leptospiren. Viele haben sich also in dieser Zeit geteilt.

Nachdem wir also den Apparat näher studiert hatten, haben wir eine Methode, mit welcher es möglich ist, Wasser während einer beliebigen Zeit mit einem Teil der Haut eines Meerschweinchens in Berührung zu bringen, ausgearbeitet. In dieser Weise würde es vielleicht möglich sein, die Anwesenheit von einigen wenigen Leptospiren in Wasser, beziehungsweise in dem, im obigen Apparat gesammelten Schlamm, nachzuweisen.

Wie oben angegeben, wird ein Glaszylinder, der unten an zwei Seiten etwas hohl geschliffen ist zwecks Anschluss an die gewölbte Bauchhaut des Meerschweinchens, mit Hilfe von Wachs-Paraffin und Elemiharz auf die tags vorher rasierte und gerade vor dem Versuch skarifizierte Bauchhaut des Tieres aufgeklebt (Abbildung 2). Wenn dieses Aufkleben möglichst genau gemacht wird, dann ist es absolut sicher, dass das in den Zylinder hineingelassene Wasser nicht herausfliesst. Zu diesem Zweck wird das Meerschweinchen auf ein Brett aufgespannt. Zuerst wird der Unterrand des Zylinders mit geschmolzenem Wachs-Paraffin (Paraffinum solidum 56°, 30 gr; gelbes Wachs, 10 gr; Vaselinum, 1 gr) in Berührung gebracht und schnell auf die Mitte der Bauchhaut gebracht. Dann wird eben flüssiges Wachs-Paraffin mittels

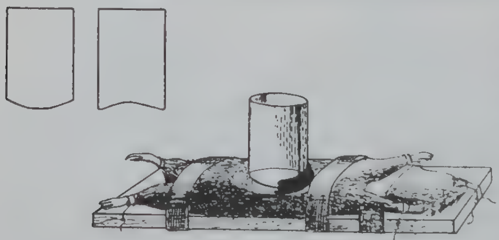

Fig. 2.

einer Pipette rings um den Unterrand des Zylinders herum gebracht und weiter auf dieselbe Stelle kleine Stücke Elemiharz. Mittels eines umgebogenen Metallstäbchens, das in der Flamme erwärmt ist, wird das Harz geschmolzen, das dann mit dem Paraffin zusammenschmilzt. Schliesslich wird ein Ring von flüssigem Wachs-Paraffin mit einer Pipette an den Unterrand des Zylinderinnern angebracht. Nach einigen Minuten kann die auf die Anwesenheit von Weilleptospiren zu untersuchende Flüssigkeit in den Zylinder hineingegossen werden.

Bevor die definitiven Versuche mit dem Apparat gemacht werden konnten, wurde untersucht, ob es möglich sei, eine einzige pathogene Leptospire im zu untersuchenden Wasser nachzuweisen. Zu diesem Zweck wurde eine Kultur von virulenten *L. icterohaemorrhagiae* bis auf eine in der Platinöse verdünnt. Eine solche unter dem Mikroskop mit schwacher Vergrösserung ohne Deckglas kontrollierte Oese wurde dann vom Objektträger in das Zylinderwasser hineingespült. Die in der Tabelle II erwähnten Versuche zeigen, dass eine einzige pathogene Leptospire sich in dieser Weise nachweisen lässt.

Tabelle II.

Menge Wasser im Zylinder	Anzahl Leptospiren in diesem Wasser	Reaktion der Meerschweinchen		
		Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet
25 ccm	1	0	2	0
25 ccm	1	1	1	0
12.5 ccm	1	1	1	0

Weil es für eine Leptospire sehr wahrscheinlich leichter ist ein Meerschweinchen aus 12.5 ccm Wasser als aus 25 ccm Wasser zu infizieren, ist in den nachfolgenden definitiven Versuchen immer nur 12.5 ccm Wasser oder Schlamm pro Zylinder untersucht worden, sodass für jeden Zentrifugierversuch zwei Meerschweinchen verwendet worden sind.

Beim definitiven Versuch wurde in Grachtenwasser, das nur durch Drahtgaze (sogenannte Benzingaze, nicht Filtrierpapier, Glaswolle oder Watte) filtriert war, eine möglichst genau (wie oben angegeben) abgezählte Menge von virulenten pathogenen Leptospiren gespült. Weil Appelman (1) mit seiner Methode eine Leptospire in 3 ccm Wasser nachweisen konnte, d.h. 1667 Leptospiren in 5 Liter Wasser, haben wir im ersten Versuch ebenfalls 1000 Leptospiren 5 Liter Wasser zugesetzt. Es war ja unsere Absicht die APPELMANsche Methode zu verbessern. In weiteren Versuchen ist die Anzahl Leptospiren, die dieser Wassermenge zugefügt wurde, vermindert.

Das schlammige Zentrifugat wurde in 25 ccm Wasser gesammelt und über zwei, auf der Bauchhaut von zwei Meerschweinchen angebrachte, Zylinder verteilt. Im Winter wurde das Grachtenwasser bevor der Zentrifugierung auf 20°C. in einem Wasserbade erhitzt. Sieh Tabelle III.

Tabelle III.

Versuche mit der Zentrifugalmethode.

Anzahl Versuche	Wassermenge, 1 Leptospire enthaltend	Reaktion der Meerschweinchen		
		Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet
1	5 ccm	2	0	0
1	17 ccm	1	0	1
4	25 ccm	0	6	2
1	50 ccm	2	0	0
1	90 ccm	0	1	1
1	110 ccm	0	1	1
1	125 ccm	0	1	1

Resultat: Mit der Zentrifugalmethode ist es unbedingt möglich 1 Leptospire in 20 ccm Wasser nachzuweisen.

b. Die Strömungsmethode.

Indem der Schlamm im Zentrifugat bei der unter *a.* beschriebenen Zentrifugalmethode selbstverständlich das hineindringen der Leptospiren in die Meerschweinchenbauchhaut hemmt, haben wir versucht eine andere Methode auszuarbeiten, in welcher der nachteilige Einfluss des Schlamms vermieden wird, jedoch unter Aufrechterhaltung eines innigen Kontaktes mit der Tierhaut.

Bei der Strömungsmethode lässt man das zu untersuchende Wasser zwei Stunden langsam über die rasierte und skarifizierte Bauchhaut eines Meerschweinchens hinfliessen.

Zu diesem Zweck wird in die sub *a.* beschriebene Weise ein Glaszylinder angebracht, der dicht am unteren Rande eine Glastülle enthält, durch die das Wasser, das aus einem Heber sehr langsam in den Glaszylinder tropft, wieder herausfliessen kann (Abbildung 3). Es bildet sich also im Zylinder eine etwa 0.5 cm hohe Wasserschicht. Das zu untersuchende Wasser, das von Zeit zu Zeit mit einem Rührlöffel behandelt wird, wird in einem Wasserbade auf 30°C. erhitzt, namentlich auf die Temperatur des Wassers, in dem APPELMAN seine Meerschweinchen badete.

Lässt man das Wasser möglichst langsam in den Zylinder hineintropfen, so dass die Leptospiren am leichtesten das Meerschweinchen infizieren können, so werden auf je ein Meerschweinchen etwa 200 ccm Wasser untersucht.

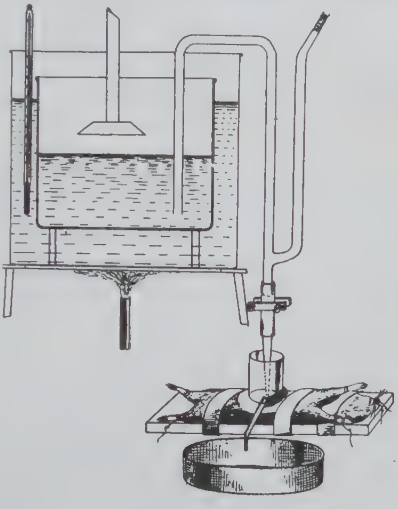

Fig. 3.

Die Versuche findet man in der Tabelle IV.

Resultat: Die Möglichkeit 1 Leptospire in 25 ccm Wasser nachzuweisen ist hier grösser als mit der Zentrifugalmethode. Selbst ist es möglich mit der Strömungsmethode 1 Leptospire in 50 ccm Wasser nachzuweisen.

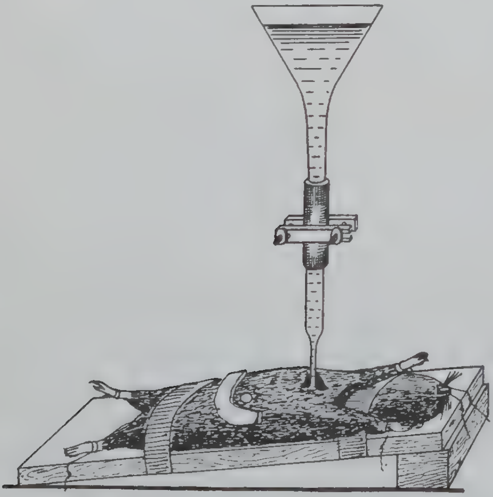

Fig. 4.

c. Die subkutane Durchströmungsmethode.

Obgleich besonders bei der sub *b.* beschriebenen Methode der Kontakt der Meerschweinchenhaut mit eventuell im Wasser sich findenden Leptospiren ziemlich gross ist, haben wir versucht, diesen Kontakt dennoch zu vergrössern.

In die rasierte Bauchhaut eines auf einem Brett aufgespannten Meerschweinchens wird mittels einer stumpfen geschlossenen Schere ein 4 cm langer subkutaner

Tabelle IV.

Versuche mit der Strömungsmethode.

Anzahl Versuche	Art des Wasser	Wassermenge, 1 Leptospire enthaltend	Durchschnittliche durchgeströmte Wassermenge	Anzahl durchgeströmter Leptospiren	Reaktion der Meerschweinchen		
					Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet
2	Leitungswasser	25 ccm	2000 ccm	80	2	2	0
1	,,	25 ccm	250 ccm	10	1	1	0
1	,,	50 ccm	2500 ccm	50	0	2	0
3	,,	50 ccm	200 ccm	4	4	2	0
1	Oberflächenwasser	50 ccm	170 ccm	4	1	1	0
1	,,	90 ccm	210 ccm	2 — 3	0	1	0
2	Leitungswasser	100 ccm	2000 ccm	20	0	5	0
1	,,	100 ccm	275 ccm	2 — 3	0	2	0
1	Oberflächenwasser	110 ccm	165 ccm	1 — 2	0	2	0
1	,,	125 ccm	200 ccm	1 — 2	0	1	0

Longitudinalkanal gemacht. Auf der einen Seite dieses Kanals ist eine enge Glasröhre (nicht zu kleine Oeffnung) befestigt, die mit einem Trichter, in dem sich das zu untersuchende Wasser befindet, in Verbindung steht. Die andere Seite des Kanals enthält eine ungefähr 6 mm weite Oeffnung, durch welche das unter der Haut durchströmende Wasser abfliessen kann (Abbildung 4).

Die Befestigung der engen Glasröhre muss möglichst genau stattfinden. Zuerst werden um die Umbiegungsstelle dieser Röhre herum, wo sie in den Longitudinalkanal eintritt, schmale Streifchen Leukoplast geklebt, die dann mit warmem flüssigen Wachs-Paraffin übergossen werden. Dann wird Elemiharz in genau derselben Weise wie sub *a*. beschrieben ist aufgebracht.

Die Strömungsgeschwindigkeit wird so gewählt, dass 100 ccm Wasser in 2 bis 2.5 Stunden durchfliessen. Unter die Ausströmungsöffnung des subkutanen Kanals wird ein Wattebausch gelegt zur Aufsaugung des ausströmenden Wassers. Dieser Bausch muss regelmässig erneuert werden, denn es ist sehr wichtig, die Berührung der Rückenhaut des Tieres mit Wasser vorzubeugen. Trägt man keine Sorge dazu, dann wird das Meerschweinchen schwerlich die Probe bestehen.

Die zu untersuchende 100 ccm Wasser werden fraktioniert zugegossen. Vorher wird das Wasser in einem Wasserbade auf 30°C. erhitzt.

In der Tabelle V sind die Versuche erwähnt.

Resultat: Auch mit dieser Methode kann 1 Leptospire in 50 ccm Wasser nachgewiesen werden. Hier erreicht sich wahrscheinlich die grösste Möglichkeit dazu.

d. Vergleichende Untersuchungen mit den drei beschriebenen Methoden.

Selbstverständlich kann man den Wert der drei oben beschriebenen Methoden am besten beurteilen, wenn das gleiche Wasser, dieser drei Methoden gemäss, auf die Anwesenheit von Leptospiren untersucht wird. Dies lässt sich nur machen, wenn die kleinst mögliche Leptospirenzahl, obwohl vielleicht nach bloss einer der drei Methoden nachzuweisen, angewendet wird.

Die Ergebnisse dieser Versuche sind in der Tabelle VI zusammengestellt. Der letzterwähnte Versuch wurde mit natürlichem Grachtenwasser, das einige Monate vorher mittels der Strömungsmethode ein positives Resultat ergeben hatte, angestellt (sieh sub *e*).

Resultat: Die subkutane Durchströmungsmethode scheint die empfindlichste der drei Methoden zu sein.

Tabelle V.

Versuche mit der subkutanen Durchströmungsmethode.

Anzahl Versuche	Art des Wassers	Wassermenge, 1 Leptospire enthaltend	Anzahl durchgeströmter Leptospiren	Reaktion der Meerschweinchen		
				Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet
1	Oberflächenwasser	25 ccm	4	1	0	0
1	,,	50 ccm	2	1	0	0
1	,,	75 ccm	1 — 2	1	0	0
3	,,	100 ccm	1	0	3	1
1	Leitungswasser	100 ccm	1	0	1	0

Tabelle VI.

Versuche mit den drei Methoden.

Wassermenge, 1 Leptospire enthaltend	Versuche mittels der								
	Zentrifugalmethode			Strömungsmethode			Subkutanen Durchströmungsmethode		
	Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet	Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet	Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet
50 ccm	2	0	0	1	1	0	1	0	0
90 ccm	0	1	1	0	1	0	1	0	0
110 ccm	0	1	1	0	2	0	0	1	0
125 ccm	0	1	1	0	1	0	0	1	0
Uiterste Gracht	0	2	0	0	2	0	0	1	0

e. Versuche mit natürlichem Oberflächenwasser.

Indem die Zentrifugalmethode für praktische Anwendung zu grosse Schwierigkeiten bietet (sieh sub *f*, sind noch 10 Wasserproben [1]) von natürlichem Oberflächenwasser, die mehr oder weniger Weilverdächtig waren, nur mittels beider anderen Methoden untersucht worden (Tabelle VII).

Tabelle VII.

Untersuchung von natürlichem Oberflächenwasser.

Anzahl Versuche	Versuche mittels der					
	Strömungsmethode			Subkutanen Durchströmungsmethode		
	Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet	Anzahl positiv	Anzahl negativ	Anzahl interkurrent verendet
10	1	9	0	0	9	1

Resultat: Nur das Wasser des „Uiterste Grachts" wurde bloss mittels der Strömungsmethode positiv befunden. Am 1 Januar 1940 war ein 70-jähriger Mann zur Stelle, wo das Wassermuster genommen wurde, ins Wasser gefallen. Ungefähr 10 Tage später war er Weilkrank geworden. Am 9 Mei 1940 wurde das Wasser, mit positivem Erfolg untersucht. Es hat sich gezeigt, dass eine *Leptospira icterohaemorrhagiae* von der vollständigen Biotype (nach GISPEN und SCHÜFFNER (3)) das Meerschweinchen infizierte. Am 25 Oktober 1940 konnte aber mittels der drei Methoden kein positives Resultat erreicht werden (sieh sub *d*). Das Wasser des Nieuwe Rijn, das APPELMAN am 28 März 1933 positiv ergibt, war jetzt also negativ befunden. Dieses kann einen nicht wunder nehmen, denn die sich am Wasser findende Fabrik, wo sich damals viele Ratten aufhielten, bestand seit mehreren Jahren nicht mehr.

f. Schlussfolgerungen.

1. Obwohl die Zentrifugalmethode im Vergleich mit der Bade-

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[1]) Uiterste Gracht, Groenhazengracht, Nieuwe Rijn, Rapenburg, Galgewater, Rijn en Schiekanaal, Zijl, Poelmeer, Plasvijver, Wiesengraben.

methode APPELMANS eine Verbesserung bringt, kann sie auf folgenden Gründen nicht empfohlen werden. 1) Die Methode erfordert einen teuren Apparat; 2) Die Meerschweinchen sterben durch den Kontakt mit dem schlammigen Zentrifugat oft interkurrent an einer Sepsis und 3) Der bald im Zylinder sich absetzende Schlamm verhindert das Hineindringen der Leptospiren in die Haut der Meerschweinchen.

2. Bei der Strömungsmethode oder bei der subkutanen Durchströmungsmethode genügt ein Meerschweinchen zum Nachweis von 1 Leptospire in 25 ccm Wasser.

3. Stehen 2 Meerschweinchen für die Untersuchung zur Verfügung, dann benutzt man am besten beide sub *b*. und *c*. erwähnten Methoden nebeneinander. Es besteht dann die Möglichkeit 1 Leptospire in 50 ccm Wasser nachzuweisen.

4. Wünscht man 1 Leptospire in 50 ccm Wasser mit Sicherkeit nachzuweisen, dann verwende man sowohl für die Strömungsmethode, wie für die subkutane Durchströmungsmethode besser 2 Meerschweinchen.

5. Gegenüber der APPELMANschen Bademethode geben die letztgenannten Methoden die folgenden Vorteile: *a*) eine 8—17 Mal grössere Möglichkeit pathogene Leptospiren in Oberflächenwasser nachzuweisen; *b*) eine geringere für den Versuch erforderliche Anzahl der Meerschweinchen.

6. In den Versuchen wurden die Meerschweinchen durchaus nicht immer mit den Weilleptospiren infiziert, obwohl Leptospiren enthaltendes Wasser mit der verwundeten Haut in Berührung gebracht wurde. Dies stimmt mit der Erfahrung, dass man die Weilsche Krankheit nicht leicht bekommt, überein.

7. Der Infektionsgrad des Oberflächenwassers mit Weilleptospiren ist doch geringer als wir früher (APPELMAN und VAN THIEL (2)) kraft des Fundes von Leptospiren (1 Leptospire in 3 ccm Wasser) mittels der Bademethode im Nieuwe Rijn meinten.

8. Benutzung einer grösseren Menge Meerschweinchen war aus Ersparungsgründen nicht möglich.

Zusammenfassung.

Es wurde versucht die Bademethode APPELMANS zwecks der Isolierung von pathogenen Leptospiren aus Oberflächenwasser zu verbessern.

Drei neue biologische Methoden sind beschrieben worden: eine

Zentrifugalmethode, eine Strömungsmethode und eine subkutane Durchströmungsmethode. Namentlich die beiden letzten Methoden werden empfohlen. In dieser Weise ist es möglich eine einzige Leptospire in 50 ccm Wasser nachzuweisen.

Der Bademethode gegenüber ergeben letztgenannten Methoden die folgenden Vorteile: *a*) eine 8—17 Mal grössere Möglichkeit pathogene Leptospiren in Oberflächenwasser nachzuweisen; *b*) eine geringere für den Versuch erforderliche Anzahl der Meerschweinchen.

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INVESTIGATIONS ON THE OCCURRENCE OF BACTERIOPHAGE-FREE SPORES OF *BACILLUS MEGATHERIUM* 899 OF DEN DOOREN DE JONG

by

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(Received October 15, 1941).

In 1931 DEN DOOREN DE JONG isolated a strain of *Bac. megatherium* which proved lysogenic (1). The bacteriophage produced by this strain had the property to lyse a strain of *Bac. megatherium* 338 b′ which had been isolated by the same author and had been transformed into a non-sporogenic, lysosensitive variation by means which are described in the publication. Moreover DEN DOOREN DE JONG had found, that *Bac. megatherium* 899 is able to produce the phage even after having been heated to 70° C., a temperature which the spores can stand, but which inactivates the phage and kills the vegetative forms of the bacteria.

DEN DOOREN DE JONG originally considered this as a certain proof that the phage is specific for the bacterium it is contained by and that it is produced by the latter independent of exterior conditions. This conception, however, has been conclusively confuted by other investigators, who claim, that the bacteriophage is non-specific for the bacterium it lyses as well as for the bacterium it is contained by, and that it belongs to the class of ultravira. FLU (2) gave a satisfactory explanation of the phenomenon described by DEN DOOREN DE JONG by assuming that the influence which protects bacteria in their sporulated condition against unfavourable outward conditions might at the same time protect the phage, which had penetrated into the bacteria, against high temperature. He considers the low water content of the spores as such an eventual protecting influence.

In case the latter hypothesis is right, and till now there are no convincing arguments to tell against it, it might be possible for spores, which are not infected with the phage, to occur because it is to be supposed that not all of the spores will be infected with the phage.

Mr. and Mrs. WOLLMAN (4) tried to prove, that each spore is able to produce one single phage. The experiments of the couple however have sustained a lot of criticism, among others from GRATIA (3) and from FLU (2), who proved that the conclusion they drew from the results of their experiments were unfounded, faults having occurred in the technique applied by them.

FLU (2) furnished proof, that a culture of *B. megatherium* may contain more spores than phage particles. He worked with the strain 338 b' of DEN DOŎREN DE JONG, which had been rendered lysogenic by special treatment.

In accordance with this result it might be theoretically possible to isolate spores from *B. megatherium* 899 which would not produce phage and by these means arrive at the production of a strain of *B. megatherium* 899 free from phage. The following experiments were put up in order to study this question. During the course of the researches I was faced by other problems that will be dealt with along with the description of the proceedings.

Usually the bacteria were suspended in broth. Whenever this offered difficulties I suspended in water; 0.9% NaCl having an unfavourable influence on phage and bacteria which cannot be figured out.

Initially I made single-cell cultures with the apparatus of CHAMBERS.

A description of this apparatus which is based on the instrument originally constructed by SCHOUTEN, will not be necessary, as it has already found application everywhere in bacteriology.

To start with, an 8 days old broth culture of *B. megatherium* 899, in which there occurred already a great many spores, was heated to 80° C. during 15 minutes. From this heated suspension single-cell cultures were made. After some practice it proved quite well possible to distinguish in the illuminated field the spores from the dead vegetative forms. The results of this work however were disappointing. Out of 100 single-cell cultures only two did grow and both of them produced phage. To exclude any technical error — it might be conceived that the isolated spores had been brought under unfavourable conditions — the experiment was repeated with a non-heated suspension of *B. megatherium* 899. From the 24 isolated spores, 23 developed into cultures. So presumably I may claim that no technical error has been made. The only possible explanation is, that the greater part of the spores does not survive the temperature of 80° C.

In order to look further into this matter the following was done: An

8 days old broth culture was heated to 80° C. during 15 minutes. After the suspension had been provided with glass beads, the suspension was kept for half an hour in a shaking apparatus, in order to destroy as much as possible the chains of bacteria which are produced by *B. megatherium*. Subsequently the number of spores in the suspension was counted, by means of the counting chamber of PETROFF HAUSSER.

It appeared that an old culture of *B. megatherium*, which contains a great many spores, after a longer shaking will contain markedly fewer chains than a young culture, such as has been used by FLU and by GRATIA in their experiments. Apparently as a result of the spore formation connections in the chains are less solid. So whilst in young cultures a predomination of chains can be noted, in this latter case we found to a large extent single spores. Still we must not attach too great a quantitative value to the results of this experiment and only very clear cut results may count.

Following this the suspension was streaked over agar plates from different dilutions. After 48 hours at 37° C. the colonies on the plates were counted and now it was comparatively easy to calculate what percentage of spores had developed into colonies. This procedure was repeated 3 times and it was found that respectively 0.9%, 1% and 1.6% of the spores had produced colonies. This agrees with the result of the single-cell cultures.

This result showed clearly the insuitability of the method. When some conclusion bearing on the occurrence of *B. megatherium* spores without phage is to be arrived at, the isolation of a great many spores to be tested for phage is needed. As merely 1% of the spores isolated appeared to be alive such an investigation would take years and would be impossible for a single person to accomplish. So I gave up this method.

It was now tried to arrive at a result by other means. An 8 days old broth culture of *B. megatherium* was heated during 15 minutes to 80° C. and shaken for half an hour with glass beads. Now a loopful was streaked over agar plates. The isolated colonies which had developed were investigated for the occurrence of phage. A 100 colonies were tested and all of them contained phage.

Subsequently a suspension was made in 0.1 n HCl from an 8 days old agar culture of the bacteria. It seemed to me undesirable to add HCl to the broth, because the broth has a fairly strong buffering power. The bacteria were left in the acid during one hour; it is known that HCl in this concentration kills the phage as well as the vegetative forms of *B. megatherium*. After this the acid was disposed of by centrifugation. The bacteria were subsequently washed three times with distilled water and after having been brought into suspension again they were kept in the shaking apparatus for half an hour and streaked on agar plates. Again isolated colonies were investigated for the occurrence of phage.

A 100 colonies were examined and all of them contained phage. As a matter of fact in the latter two methods one is not sure to have worked with single-cell cultures, but in any case some of the colonies will have grown from single cells.

Discussion and conclusion.

These experiments make it clear that it is not an easy matter to isolate a spore which is unable to produce phage. To conclude for the non-existence of such spores, however, would be inexact. Neither would one be justified, in case it had been possible to find such a spore, to deduce that the theory of DEN DOOREN DE JONG were unfounded. In such a case it might have been possible for spores which would not produce phage to be variations of the normal kind. So neither may it be concluded now that the theory of DEN DOOREN DE JONG is founded and that the phage is produced by the bacteria itself, an actual isolation of phage-free spores having proved impossible.

It may be conceived that the spores which contain phage do possess an exceptional resistance and that they are therefore also the ones that tolerate the treatment with heat or acid.

So a method is to be looked for which might leave a greater part of spores alive, whereas the phage and the vegetative forms are killed. It is quite possible that when such a method were applied, a greater number of spores might be found, that would not contain phage. A treatment that leaves only 1% of the spores alive is in fact much too radical and it furnishes cultures from spores, that have been selected by very strong means indeed. One can expect, however, with certainty, that the number of spores that do not contain phage, will be small, because otherwise it cannot be explained thaf during the many years in which experimentators worked with *B. megatherium* 899, there has never been found a strain, free from phage. The only conclusion to be drawn from these results is, that it is not possible by the methods I tried, to isolate a *B. megatherium* strain 899, that does not contain phage. It leaves the way open however for a method that may actually lead to it.

Summary.

It has been tried to isolate from *B. megatherium* strain 899 from den Dooren de Jong a spore that does not contain phage. This has failed. Moreover it has been proved, that in case a suspension containing spores is heated to 80° C., not all the spores are resistent to this temperature as one would expect, but only about 1%. It is left open, whether phage-free spores may occur.

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AU SUJET DE LA FERMENTATION DU GLUCOSE PAR *PL. TETANI*

par

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(Reçu le 19 décembre 1941).

Dans un article récent TASMAN et PONDMAN (5) viennent de mettre en doute les résultats que nous avons publiés avec nos collaborateurs KIRCHHEINER, BOORSMA et VEILLON (1, 4). Nous croyons au contraire que ces auteurs ont confirmé et même amplifié nos résultats, et qu'il s'agit simplement d'une divergence d'interprétation des faits.

1°) TASMAN et PONDMAN ont réussi à faire fermenter 100% de glucose par la souche 132 PRIGGE Frankfort, souche qu'ils croient être la souche L, dont nous nous sommes servis. Or nous croyons que cette souche n'est pas la souche L, car nous n'avons jamais envoyé notre souche à M. PRIGGE, Frankfort. D'ailleurs les souches ne changent pas de nom. Si notre souche est parvenue à Frankfort, par une voie indirecte, elle doit toujours s'appeler L. Ceci porte donc vraisemblablement à 2 les souches qui font fermenter le glucose. La nôtre, L, qui fait fermenter 4,39 g. de glucose en moyenne par litre et la souche 132 qui fait fermenter jusqu'à 15 et 20 g. par litre, chose que nous n'avons jamais constatée avec la souche L en 2 années d'expérience (portant sur des productions de 200 litres de toxine par semaine).

2°) TASMAN et PONDMAN prétendent que la souche L est impure, et n'apportent aucune preuve à cette assertion. Ils ont aperçu dans la souche 132 des filaments longs et minces, à GRAM-négatif. Ceci est très fréquent chez les anaérobies toxigènes, qu'il s'agisse de *Pl. tetani*, de *W. perfringens*, de *Cl. septicum*, de *Cl. sordellii*, de *Cl. oedematiens* etc. etc. Ces anaérobies, en effet, ont une évolution de population extrêmement rapide. Ils atteignent la phase de mortalité logarithmique souvent en 2 ou 3 jours et parfois même en 24 h. Les cultures sont remplies de formes longues asporulées et GRAM-négatives qui sont des formes végétatives mortes. Ceci explique l'échec complet de Tasman et Pondman dans leurs essais de repiquage de ces formes. Ces auteurs ont essayé de repiquer des cadavres. Or les cadavres, même au micromanipulateur, ne repoussent plus. Par contre, ils ont repiqué au micromanipulateur des formes sporulées authentiquement tétaniques qui ne font pas fermenter le glucose. Ceci est un fait appartenant à un ordre de choses bien connu et très classique. *Pl. tetani* est un des anaérobies se dissociant le plus facilement et le plus fréquemment et nous avons étudié ce fait classique après bien d'autres auteurs, en particulier Condréa. Quand on manipule sans certaines précautions le *Pl. tetani*, il émet sans discontinuer des variantes qui perdent, entre autre chose, leur faculté de faire fermenter le glucose. Ces variantes se reconnaissent macroscopiquement en gélose profonde où elles forment de petites colonies à potentiel oxydoréducteur très faible (2). Elles ne sont même plus capables de réduire l'indigocarmin. Il y a perte de fonction, probablement perte de réductase ou de déshydrogénase, et en général ces petites colonies finissent par donner des souches oligotoxigènes, et incapables de faire fermenter le glucose. La souche L, comme beaucoup d'autres, quand elle est entretenue sur un bouillon quelconque fournit des quantités énormes de ces variantes. C'est pourquoi nous avons eu recours au procédé de bouillon de cervelle acide, vidé d'oxygène à l'aide de la pompe Cenco Hyvac qui donne le 0.1 de mm de Hg. Dans ces conditions l'apparition des variantes oligotoxigènes et oligoenzymatiques devient rare (3).

Ce sont ces variantes que Tasman et Pondman ont repiqué au micromanipulateur; en effet, cette manoeuvre, qui expose le *Pl. tetani* à l'oxygénation, le prédispose à donner des variantes. C'est pourquoi la nouvelle souche qu'ils en retirent ne fait plus fermenter le glucose.

3°) La présence de méthanol dans les produits de fermentation du bouillon glucosé par *Pl. tetani* est en effet surprenante. Nous en avons été surpris les premiers et nous comprenons très bien que Tasman et Pondman le furent aussi. Pas plus qu'eux, nous ne nous l'expliquons et nous nous sommes bornés à la constater. Comme eux nous pensons que de nouvelles recherches sont nécessaires pour l'expliquer.

Résumé et conclusions.

Les recherches de Tasman et Pondman confirment et amplifient les nôtres. La souche 132 fait fermenter 100% de glucose et donne des variantes qui ne font plus fermenter le glucose comme toutes les souches de *Pl. tetani*, qui ont été étudiées avant eux et sous cet angle. Les filaments longs et Gram-négatifs qu'ils ont pris pour une contamination sont des cadavres de bactéries dont la population est arrivée à la phase de mortalité logarithmique.

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SUR LA FERMENTATION DE LA GLUCOSE PAR *CL. TETANI*

par

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(Reçu le 24 décembre 1941).

Nous avons constaté que *Cl. tetani* est incapable de fermenter la glucose (4), ce qui est contraire aux recherches de PRÉVOT et KIRCHHEINER (2) et de BOORSMA, PRÉVOT et VEILLON (1). PRÉVOT et BOORSMA (3) croient pouvoir défendre leur point de vue dans l'article précédent. Qu'il nous soit permis de donner ici encore un bref exposé de notre opinion et de répondre à quelques remarques de ces auteurs.

Nous n'avons pas réussi à faire fermenter la glucose par les souches tétaniques ,,12'', ,,Utrecht'' et ,,Kopenhagen'' que nous avions à notre disposition. Du reste les souches nommées se conduisent morphologiquement et en culture parfaitement comme les souches tétaniques ,,normales''; la production de la toxine par ces souches est bonne aussi.

Nous avons reçu de M. PRIGGE de Frankfort s/Main la culture ,,Tetanus 132'', qui était selon M. PRIGGE un repiquage de la souche ,,L'' de PRÉVOT et de ces collaborateurs. La raison que nous ne nous sommes pas adressés directement à M. PRÉVOT fut que nous n'avons pas pu obtenir un contact avec Paris à ce moment-là (juillet 1940). Or, de cette culture ,,Tetanus 132'' nous avons réussi à isoler une souche tétanique d'après notre opinion ,,normale'', qui était elle aussi incapable de fermenter la glucose. Mais un changement des souches reste toujours possible et à l'avenir nous répéterons sûrement notre recherche avec la souche ,,L'' reçue directement de M. PRÉVOT.

L'explication que PRÉVOT et BOORSMA donnent de l'échec de nos efforts pour isoler les formes longues et GRAM-négatives — une impureté du ,,vrai'' *Cl. tetani* 132 —, peut être juste, mais elle n'a pas été démontrée. D'ailleurs cette question n'est pas de grand intérêt.

Ce qui est plus important c'est que selon nous PRÉVOT et BOORSMA tournent les choses. D'après ces auteurs toutes les souches tétaniques, qui ne sont pas capables de fermenter la glucose, mais se conduisent morphologiquement et en culture parfaitement selon l'opinion classique — c'est à dire, qu'elles montrent sous la microscope des formes courtes, Gram-positives, formant en abondance des spores et produisant une toxine forte —, appartiendraient aux souches „anormales" et bien uniquement, parce qu'elles ne fermentent pas la glucose. Cette opinion est en contradiction avec celle de la majorité des auteurs et elle nous semble intenable.

Prévot et Boorsma disent que nous n'avons pas donné une seule preuve pour confirmer notre opinion que la souche 132 était impure. Cependant le fait doit suffire que dans cette culture des microbes longues, Gram-négatives, non sporulants et d'une culture très difficile apparaissent à côté des bacilles tétaniques, qui seraient d'après nous „normales". Et si nous n'avons pas réussi à isoler cette impureté en culture pure, les résultats de nos recherches ne perdent pas de leur valeur.

Conclusion.

Selon nous nos recherches ne forment ni une confirmation ni une amplification de l'observation de Prévot et Boorsma, mais nous combattons les conclusions que ces auteurs ont tirées de leurs recherches. La souche 132 est impure sans parler de son origine. Il en suit que la faculté de cette souche de fermenter la glucose manque de toute force de démonstration à l'égard de la question posée.

Conformément à la grande majorité des auteurs qui travaillent dans ce domaine nous sommes toujours d'avis que les souches tétaniques normales ne fermentent pas la glucose.

Références.

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ABSTRACTS

J. VAN BEYNUM en J. W. PETTE, Een methode voor het aantoonen van boterzuurbacteriën, speciaal geschikt voor het onderzoek van melk (Eine Methode zum Nachweis von Buttersäurebakterien, besonders geeignet für die Milchuntersuchung). Versl. van landbk. onderz. **46**, 379, 1940. 's-Gravenhage, Algemeene Landsdrukkerij. Mit Zusammenfassung in deutscher und englischer Sprache.

Für den Nachweis von Buttersäurebakterien wird eine Gärungsprobe benutzt mit Milch als Nährboden. Um auch *Cl. tyrobutyricum* (das käsegefährliche Buttersäurebakterium) nachweisen zu können, musz 0,5% Traubenzucker zugesetzt werden und um das Wachstum von anderen Bakterien genügend zu hemmen wird pro 50 ml Milch 1 ml n/1 Säure (z.B. Salzsäure) zugesetzt. Nach der Impfung wird pasteurisiert und anaerob gezüchtet in V-förmigen Glasröhrchen, welche an einer Seite geschlossen sind mit Gummistöpsel. Züchtung bei 35°—40° C. Die Beurteilung (Gasbildung und Geruch) hat am 3. Tage zu geschehen.

Weil der gebrauchte Nährboden hauptsächlich aus Milch besteht, kann man für die Untersuchung von Milch die zu untersuchende Milch selbst als Nährboden verwenden (4 V-Röhrchen mit je 10 ml).

v. B.

J VAN BEYNUM en J. W. PETTE, De invloed van de voeding van het vee op de besmetting der melk met boterzuurbacteriën (Einflusz der Fütterung der Kühe auf den Buttersäurebakteriengehalt der Milch). Versl. van landbk. onderz. **46**, 397, 1940. 's-Gravenhage, Algemeene Landsdrukkerij. Mit Zusammenfassung in deutscher und englischer Sprache.

Mit Hilfe der im vorigen Artikel beschriebenen Buttersäurebakterienprobe wurde während langerer Zeit die Milch eines Viehbestandes untersucht. Eine starke Verunreinigung mit diesen Bakterien war anzunehmen wenn von 4 Parallelröhrchen mit je 10 ml Milch 3 oder 4 Buttersäuregärung hatten. Wenn die Kühe mit Heu oder künstlich getrocknetem Gras gefüttert wurden, war der Ausfall der Probe fast stets negativ. Auch während des Sommers (Kühe auf den Wiesen) war die Milch buttersäurebakterienfrei. Bei Fütterung mit buttersäurebakterienhaltiger Silage enthielt die Milch viele Buttersäurebakterien

Nach Beendung der Fütterung mit buttersäurebakterienhaltiger Silage verschwinden die Buttersäurebakterien allmählig aus der Milch, aber beim untersuchten Betrieb dauerte es ungefähr 3 Wochen bevor die Milch frei von Buttersäurebakterien war. Eine grafische Darstellung erläutert den Text.

v. B.

C. I. Kruisheer, P. C. den Herder, W. C. Smit en A. de Haan, Het bacteriologisch-chemisch kwaliteitsonderzoek der Nederlandsche keuringsboter (Die bakteriologisch-chemische Qualitätsprüfung niederländischer Butterproben). 's-Gravenhage, Algemeene Landsdrukkerij 1940.

Von ungefähr 1000 Butterproben des Butterqualitätsbüros (Z.K.B.) wurde studiert ob die Sinnesprüfung, ausgeführt von erfahrenen Butterprüfern, Uebereinstimmung zeigte mit den Untersuchungsergebnissen des Laboratoriums. Alle Butter war bereitet aus pasteurisiertem Rahm und wurde vor der Reifung 7 Tage aufbewahrt bei 13° C. Es wurden bestimmt: Katalasezahl, Gesamtzahl Mikroörganismen auf Kaseinagar, die Anzahl Schimmelpilze, Hefen, fettspaltende und eiweiszspaltende Mikroörganismen, Coliähnliche, Milchsäurebakterien auf Chinablaulaktoseagar und nicht-säurebildende Mikroben. Die chemischen Bestimmungen betrafen: sichtbare Wassertropfen, Kochsalz, Eisen- und Kupferverbindungen. Die Korrelationen zwischen Qualitätsklassen und Laboratoriumsbestimmungen .wurden berechnet und obwohl bei den jungen Butterproben noch keine starke Entwicklung von Geruchs- und Geschmacksfehler stattfinden konnte, war doch schon ein Zusammenhang der Qualitätsklasse mit Katalasezahl, Gesamtzahl Mikroorganismen auf Kaseinagar, Anzahl Schimmelpilze, sichtbaren Wassertropfen und den genannten Schwermetallen ersichtlich. Etwas weniger deutlich war der Zusammenhang mit Anzahl Hefen, fettspaltenden, eiweiszspaltenden und coliähnlichen Mikroörganismen. Es bestand keine Korrelation mit der Anzahl Milchsäurebakterien. Auch die Korrelationen zwischen den erhaltenen Zahlen untereinander wurden berechnet. Sommer- und Winterzahlen wurden verglichen. Es wurden Maximalwerte für gute Butter angegeben.

v. B.

M. van Riemsdijk, A. B. C. van het bacteriologisch-serologisch Laboratorium. Bacteriologische-serologische methoden en recepten, samengesteld ten behoeve van een bacteriologisch practicum. Met een voorwoord van Prof. Dr. R. H. Saltet. Vierde, belangrijk vermeerderde druk. N.V. Swets en Zeitlinger, Amsterdam 1941. Prijs ƒ 8,10 ing., ƒ 9,40 geb. (M. van Riemsdijk, A. B. C. du Laboratoire bactériologique et sérologique. Méthodes et récettes bactériologiques et sérologiques à l'usage de cours de bactériologie. Avec une préface par le Prof. Dr. R. H. Saltet. 4e édition, considérablement augmentée).

Ce manuel de la Technique bactériologique et sérologique, à l'origine un pamflet hectografié à l'usage des étudiants de médecine à Amsterdam, s'est transformé dès lors en un livre de taille considérable, célébrant sa quatrième édition. Sans doute son auteur mérite la gratitude de nombre d'étudiants, pour qui un Manuel néerlandais de bactériologie tant étendu et paraphrasié a une valeur considérable. Cette prolixité, d'autre part, a le désavantage incontestable que l'étudiant, par l'excès de détails, se laisse séduire à exécuter inopinément les prescriptions, sans se rendre compte de ce qu'il fait en réalité. Les courts chapitres intitulés ,,théories'', parsemés abondamment parmi le texte, ne suffisent pas toujours à lui apprendre les dessous des manipulations prescrites. Une explication orale est sans doute nécessaire dans la plupart des cas.

La partie consacrée à la sérologie a été profondément revisée dans cette édition, ainsi que les chapitres traitants l'examen bactériologique du lait et de l'eau. Et ainsi l'édition entière a été revisée scrupuleusement et mise au courant de la science médicale contemporaine.

Nous espérons que l'auteur, au cas d'une édition suivante, va donner son attention spéciale au language et au style de son oeuvre et qu'elle délivrera le texte d'une abondance de lettres capitales inutiles et gênantes, ce qui augmentera sans doute la valeur de ce manuel pratique.

J.S.

W. J. Bruins Slot, Infectieuse mononucleose (Infectious mononucleosis). Geneesk. Bladen uit Kliniek en Laboratorium **38**, 79, 1940.

A survey is given of the history of the development of infectious mononucleosis and its relation to the glandular fever of Pfeiffer. The identity of both diseases is doubted. It would carry too far for a

report in this journal to go into the clinical symptoms, which are amply described in the publication. A diagnosis can only be established with certainty by means of morphological and serological blood investigation. There exists a relative and an absolute mononucleosis, the latter amounting to 60 à 90% of the white blood corpuscles. Serologically it is important that agglutinins against sheep erythrocytes are formed (reaction of PAUL and BUNNELL) which cannot be absorbed by cells from the kidney of guinea pigs, but can be absorbed by cells from cow blood and can be distinguished in this manner from FORRSMAN antibodies.

The investigations of the author comprehend twenty cases of infectious mononucleosis, observed in the last seven years. They were scattered cases, slight epidemics not having been observed. The aetiology has not been ascertained as yet. According to NYFELDT (1925) and SCHMIDT and NYFELDT (1938) *B.* (*Listerella*) *monocytogenes hominis* is isolated from blood respectively spinal fluid of patients with infectious mononucleosis. The serum from some of these patients, however, did not show any increase of agglutinins against *Listerella monocytogenes*.

For clinical reasons the author claims infectious mononucleosis to be probably a virus disease.

W. B.

M. W. FUHRI SNETHLAGE, Suikerbepalingen in brood (Determination of sugar in bread). Chem. Weekblad **37,** 16, 1940.

In order to find out whether maltbread will actually contain malt or extract of malt the maltose content of the bread has been determined with various kinds of yeast.

With *Torula monosa* maltose, lactose and non-fermentable sugars were determined, with *Saccharomyces fragilis* maltose and non-fermentable sugars and with *Saccharomyces cerevisiae* lactose and non-fermentable sugars.

As the fermentation of the bread depends much upon prevailing outward conditions, it is difficult to arrive at conclusive figures. A content of at least 3.5% maltose determined on the basis of dry weight of the crumbs should be required for maltbread; conditions other than the adding of malt or malt extract however may increase the maltose content of bread.

W. B.

A. Pondman, Bloedgroepbepalingen in de praktijk (Blood group estimation in medical practice). Geneesk. Bladen uit Kliniek en Laboratorium **37**, 291, 1940.

A blood group estimation is usually a very simple procedure; sometimes however it is difficult or next to impossible to attain a decision. It is always needed to test the red corpuscles as well as the serum they are derived from. A description is given of the blood group estimation such as it has been indicated by the Rijks-Instituut voor de Volksgezondheid, Utrecht.

Difficulties may arise owing to infection, lysis, cold-agglutination, difficult demonstration of agglutinins and agglutinogens, the presence of subgroups.

As to the latter case special α or β agglutinins may be present, having a limited activity with respect to the red blood corpuscles of group A or B and which can also agglutinine the red corpuscles of the O group. To account for this the assumption is made for each red corpuscle to passess the O antigen, on the surface of which the A, B or A and B antigens occur. So the O antigen is more or less separated from the serum. When the covering is very dense an O agglutinin may be expected, this is the so-called special α or β agglutinin. The special agglutinin is harmless when present in the blood of a donor, in the blood of the receiver however it may be harmful. A thorough theoretical knowledge and a large experience should be required in the performer of blood group estimation.

W. B.